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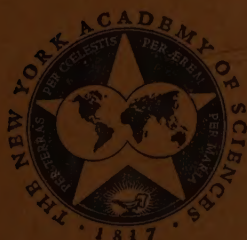
## AUTOMATIC CHEMICAL ANALYSIS

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*Consulting Editor and Conference Chairman*

RALPH H. MULLER

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\* This series of papers is the result of a conference on *Automatic Chemical Analysis* held by The New York Academy of Sciences on November 12, 13 and 14, 1959.



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## AUTOMATIC CHEMICAL ANALYSIS

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The conference on automatic chemical analysis on which this monograph is based was held only a few days after the announcement of the award of the Nobel Prize in Chemistry to Jaroslav Heyrowsky. It was thirty-five years ago that this distinguished Czechoslovakian scientist, working with Shikata, invented a machine to perform an analysis automatically. This machine recorded current-voltage curves automatically at a dropping mercury electrode, and would tell the analyst not only what substance was present, in terms of the half-wave potential, but also how much! Any learned analyst of that time would have explained that this was not only absurd but impossible. Analysis was just not done in this simple fashion, and the general idea of automatic analysis, aside from its impossibility, seemed slightly indecent.

To avoid the accusation of unwarranted cynicism, I report a personal experience illustrating the point. In 1938, more than thirteen years after Heyrowsky announced his invention, John Petras, a second-generation United States citizen of Czechoslovakian origin, and I were engaged in a study of polarography, in particular, the oscillographic presentation of polarograms. We showed that these phenomena could be displayed on a cathode ray tube screen. At about this time I was invited to address a physical chemistry colloquium at one of our renowned metropolitan universities. As my title, I chose "Polarography." Although I was an alumnus of the institution and its staff included old and respected friends, the invitation was almost withdrawn. As a result of my "take it or leave it," attitude, however, my lecture was given. Petras and I brought along about 400 pounds of equipment to take, record, and project polarograms for the audience in an attempt to demonstrate Heyrowsky's claims. The experiments were elegant and should have been convincing, but I think almost everyone in the audience felt that he was being subjected to some sort of electronic trickery. The thing that saved the day was my solemn resolve to give the complete mathematical derivation of the Ilcovic equation. This presentation was received with rapt attention and dignified approval, particularly because the equations were liberally punctuated with activity coefficients—all of which were canceled later by mutual agreement. I was congratulated on giving a very scholarly discourse and teased unmercifully for still believing that a machine could perform an analysis automatically.

It is a matter of record that Heyrowsky's visit to the United States in 1933 was frustrating and greatly discouraging to him. With a single exception,\* instrument manufacturers were indifferent to his device and most analysts and physical chemists either did not believe in or scoffed at his idea. In the hands of a few United States chemists, however, interest in his subject slowly gained momentum and, a few years later, several instrument companies capitulated and built polarographs.

\* The E. H. Sargent Co.

I have always believed that Heyrowsky committed a psychological error in developing a completely automatic polarograph so early in his studies. Most other radically new techniques are developed slowly and laboriously. They are performed manually, receive their due blessing from the theoretical pontiffs, and eventually become so well accepted and widely used that no one seriously objects to their being made automatic. Heyrowsky simply "jumped the gun." The Swedish Academy of Sciences never makes a mistake in the scientists it honors; of necessity, however, its deliberations are influenced by the attitudes and opinions of scientists in general. Thus about thirty-four years elapsed and about six thousand publications on polarography appeared before Heyrowsky could win this well-deserved reward.

One more personal reminiscence will serve to illustrate the early attitude toward automatic analysis; it is one that bears relatively directly on the topics under discussion in this monograph. About thirty-three years ago, the late Partridge and I were performing automatic photoelectric titrations. At first, the photoelectric signal at the end point was used to ring a bell. This was regarded as hilarious by analysts, so we promptly changed the arrangement so that the buret closed automatically. The hilarity did not die down, and we were regarded with raised eyebrows, at least by academicians. For at least twenty years thereafter whenever I lectured on some analytical topic in which someone was moving in the direction of automatic operations, the meeting chairman would almost invariably remark, "From what the speaker has just told us, it will not be long before the analyst can drop a sample in the hopper and the analysis will pop out, printed on paper tape." Such remarks were intended to reduce the whole matter to an absurdity or to indulge in an impossible extrapolation and, as such, always evoked roars of laughter. I was always impolite enough to add, "It should have been done long ago."

Some may think that we are living now in a more enlightened age, and that automatic operations are more rapidly and readily accepted. I am inclined to agree. However, the attitudes of an era must be compared with the contemporary state of science and technology. In view of the present astonishing resources of instrumentation, I am not too sure that our attitudes toward analysis are proportionally better than they were thirty years ago. Certainly, more people are talking and thinking about these things today, innumerable symposia and conferences are now being held on automatic methods, and publications on the subject, such as this one, appear with greater frequency.

We have elegant instruments and some of them will yield a wealth of information in a remarkably short time. Because these machines save so much time and money, it is little wonder that industrial laboratories use them wherever they can, regardless of their initial cost. The academic chemist is prone to regard automatic instruments primarily as things to be relegated to a factory where there are thousands of samples to be analyzed, where precise results are not required, and where the primary object is to save money.

Most of us, I believe, prefer a broader view. With automatic analytical instruments, we see the possibility of getting a huge amount of information in a very short time; with many of them, we can get an intimate and instantaneous picture of precisely what is going on.



I believe it can be said that, if we had three or four more techniques as precise, versatile, and perfect as chromatography and infrared spectrophotometry, the research analyst could close the books and conclude that we were well-equipped for another century of analysis. It is fortunate that we have not attained this goal, that analytical research is so fascinating, and that there are hundreds of interesting problems in the field to investigate.

The present resources of electronics, instrumentation, and computer techniques are more than adequate for the automation of any known analytical technique. Van Zandt Williams, of the Perkin-Elmer Corporation, has made some shrewd and pertinent observations on this point. He has stated that the analytical chemist must reexamine his problems in order to make his requests for instrumentation more subtle and exacting. The analyst is all too prone to ask for a simple mechanization of his current practices. On the other hand, the instrumentation expert is in a position to offer him something far better but, as a rule, he does not know enough about analytical chemistry to suggest the appropriate equipment.

As Williams has pointed out, the problem becomes very complicated when automatic analysis is extended to process control. At this stage, periodic sampling is no longer satisfactory and a continuous analysis at high speed becomes essential: a dynamic approach is required because static or intermittent sampling cannot accommodate process lags and upsets rapidly enough to make automatic control satisfactory. Moreover, high-speed computing elements become necessary if the rapidly and continuously acquired information is to be interpreted and translated into appropriate corrective action.

The information contained in this monograph on automatic chemical analysis can lighten our analytical burdens; it should also remind us, however, that there is still much to be done. Let us keep in mind that instrumentation experts can automate to almost any desired degree any new and useful discoveries in analysis. In our enthusiasm for these automatic methods, however, we should remember that, elegant as they are, they are often based on quite venerable analytical techniques.

The broad and varied techniques of chromatography afford us a fantastically critical method of separation. In many respects, it may be said that a substance is indeed pure if it is chromatographically homogeneous; gas chromatography in particular is so sensitive that serious doubts have arisen as to the availability of substances pure enough to serve as standards.

The literal meaning of the Dutch word for analysis—"the science or art of separations"—defines one of the most important aspects of analytical chemistry. We have constantly sought unique approaches to the art of separation as well as highly specific reagents and methods. If we are fortunate enough to find other methods of separation as elegant as chromatography, we can rest assured that instrumental techniques are readily available to implement them and automate them.

It is interesting to note that many contributions to this monograph deal with analyses of biological or clinical interest. It is well known that a major breakthrough occurred in the early part of this century when Folin, Benedict, van Slyke and other pioneers developed micro methods for the determination



of blood constituents. These methods served chiefly to reduce urine analysis to a secondary role and to focus attention on the clinically important information that could be derived from a reasonably economical sample of blood. Fortunately, the pediatrician has never been satisfied with a "small" sample, and we have furnished many submicro procedures in answer to his unremitting but reasonable demands. The work reported in this monograph should establish new standards of precision, economy, and efficiency for these methods. However, some important questions will still remain to be answered.

Fools and children are supposed to have an uncanny knack for asking embarrassing questions; I desire to preserve this tradition by asking such a question myself: What hopes do we have of obtaining more clinical information *in vivo*, that is, without drawing or by-passing a sample? The techniques already available for this are impressive, but I think we must admit that the biophysicist has contributed far more than the biochemist. Biophysicists have given us X-ray and nuclear techniques, the electrocardiograph and ballistocardiograph, the encephalograph, and a dozen techniques for the continuous analysis and recording of respiratory gases. The instantaneous presentation and recording of heart sounds, blood pressure, pulse rate, and temperatures have been possible for a long time. In experimental animals at least, anesthesia has been maintained automatically at any desired level for days at a time by use of the alpha wave from an encephalograph to control a servomechanism that continuously administers the anesthetic.

An essentially biochemical measurement is the anoxia measurement in which the ratio of hemoglobin to oxyhemoglobin is determined photoelectrically by means of light that can be transmitted through a septum, such as the lobe of the ear. This instrument has been so highly developed that the photoelectric signal can be used to actuate a valve releasing oxygen into the helmet of a fighter pilot long before dangerous levels of anoxia develop.

It seems quite certain that most body functions are governed from instant to instant by a host of complex chemical reactions, and that even those bioelectric phenomena that are so easily handled by instrumentation are really biochemical in origin.

Many years ago I complained to a distinguished clinical chemist that it was necessary for a person to be half dead before a complete blood chemistry analysis showed any significant changes. Far more precise analyses seemed to be required. Very patiently I was told that even among normal, healthy individuals the variations were greater than could be measured by careful analysis, and that therefore better precision was not warranted. I have never been satisfied with this answer. Everyone knows that his vitality, initiative, and feeling of well-being vary from hour to hour. We recognize mid-morning and mid-afternoon lassitude but have come up with nothing better than the coffee break. As simple a thing as the blood-sugar level can be used to measure these variations, but who wants to be punctured at half-hour intervals? The compensatory ability of the human body is indeed a divine marvel. If industrial machinery dispensed with such factors as regulators, controllers, and recording instruments and depended only on a monthly checkup, it would be a ruinous heap in two days.

Are we to hope for tricky schemes involving radio frequency absorption, magnetic susceptibilities, or nuclear magnetic resonance to give us essentially

biochemical information of clinical importance either instantly or continuously? Our older analysts keep telling us that in order to analyze, you must have a sample. In many parts of industry, samples are no longer taken, for analysis is continuous. Continuous analysis should be an ultimate aim, in the clinic as well as elsewhere.

If I am asking for the moon, and if these questions seem foolish and visionary, please remember that I have already identified myself with the kind of people who usually ask such questions.

The work reported in this monograph is of profound importance; it will contribute to the nation's health, welfare, and technological progress. In a larger sense, everyone who is interested in and contributing to automation will have to keep in mind the larger implications of automation, because some of these are explosive. At the risk of making a statement that is highly debatable in a legal or social sense, I say that a large segment of labor is pricing itself out of the market. There are some who are sufficiently indifferent and callous to say that extensive automation is the only answer to this trend; it is absurd to pay more and more for less and less and contrary to what we call progress in science and technology.

Our principal technological competitors, the scientists in Soviet Russia, have no such problem. The Soviet people are committed to the race for supremacy as a matter of national pride, if nothing else. Those among them who might protest can be shunted to less sensitive pursuits, and doubtless any who might be bold enough to act in opposition may still be dealt with even more summarily. As citizens of the United States we are circumscribed in this respect because we respect the individual, we have old-fashioned ideals, and we are a charitable folk.

Nevertheless, I believe there has been no more prophetic remark made in this century than Norbert Wiener's warning in *Cybernetics*: "We are getting to the stage where a person of mediocre ability has little to sell that is worth anyone's money to buy."

Our vast industrial output is possible because we have intricate automatic machinery. The machinery is attended by operators, but the attention required is of progressively lower order as instruments and computers replace them. Worse than this, the demand for fine craftsmanship that characterized former times is diminishing. In many respects the general decline in craftsmanship is appalling. To an increasing degree, certification of workers is based upon minimal legal requirements, union rules, obsolete seniority rules, and that perpetual abomination, experience. Experience has unquestionable value but, when it is construed as a substitute for education or sound training, it is still best defined as "that which enables one to recognize a mistake after it has been made for the third time."

Any technologist can easily extrapolate to that period at which a complete impasse will be reached. We absolutely reject a violent solution because we are people with a conscience. That this problem requires almost immediately social intelligence and planning of a high order is almost self-evident. It may suit our notions of fairness and justice to tolerate occasional and violent upsets of our industrial economy, but this tendency leaves little doubt as to the ultimate winner in the race for technical and economic supremacy.



## AUTOMATION OF ENZYME DETERMINATIONS: PHOSPHOHEXOSE ISOMERASE\*

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Current biochemical research is dependent to a great extent on the determination of the activities of various enzymes in a wide variety of experimental situations. Any new technology that would increase the rate of performance of enzyme determinations would greatly facilitate current investigations. The purpose of this and future studies is to apply automation techniques to the determination of enzyme activities. Since phosphohexose isomerase (PHI) has been studied in this laboratory in various connections,<sup>1-3</sup> it has been chosen for initial study. The automation of the analysis of this enzyme activity demonstrates several problems that may be encountered generally in enzyme automation. It is shown that the automated method for PHI is approximately eight times as rapid as the manual method, yet it retains the same precision and reproducibility.

### *Manual Method for Determination of PHI Activity*

Phosphohexose isomerase activity was determined manually by the method described by Bodansky.<sup>4</sup> This determination is based on the degree of conversion at pH 7.4 and 37° C. of 0.002 *M* glucose-6-phosphate (G-6-P) to fructose-6-phosphate (F-6-P) in 30 min. by a suitable quantity of enzyme. The PHI activity is expressed as the reciprocal of that concentration of enzyme preparation in milliliters or in grams per cubic milliliter of reaction mixture that would cause the formation of 25  $\mu$ g. of fructose as F-6-P per milliliter of reaction mixture. F-6-P is determined colorimetrically by the resorcinol-hydrochloric acid method of Roe.<sup>5</sup> The PHI method utilizes an equation that permits the expression of enzyme activity in terms of the amount of substrate changed at any stage within or beyond the zero-order portion of the reaction.<sup>6</sup> The activity is directly proportional to enzyme concentration. The principle of this method also permits the use of low initial concentrations of substrate which, as will be shown later, is important in automation of enzyme methods.

### *General Principles of Automation Analysis*

The development of the AutoAnalyzer has made available an instrument for rapid, precise colorimetric analysis of various biochemical components.<sup>7,8</sup> This apparatus has a constant-speed turntable containing places for as many as 40 fluids. Through a system of plastic tubing, a constant-flow pump aspirates

\* The work reported herein was supported in part by American Cancer Society Grants No. P-163 and P-164B, grants No. DRG 332B and 332C from the Damon Runyon Memorial Fund for Cancer Research, and by Research Grant C-4251(C1S1) from the National Cancer Institute, Public Health Service, Bethesda, Md. A preliminary report of this work was presented before the Division of Biological Chemistry of the American Chemical Society at Atlantic City, N. J., September, 1959.



these samples at stated intervals of 40, 60, or 120 sec., dilutes them if necessary, adds reagents to the samples, and segments the mixture where desired with air, which helps to regulate the rate of flow and cleans the system between specimens. The mixture of specimen and reagents is passed through various modules for such processes as dialysis, incubation, and heating, and then into a constant-flow cuvette. For the various analyses, different arrangements and bore of plastic tubing are used to introduce appropriate volumes of reagent in proper sequence.

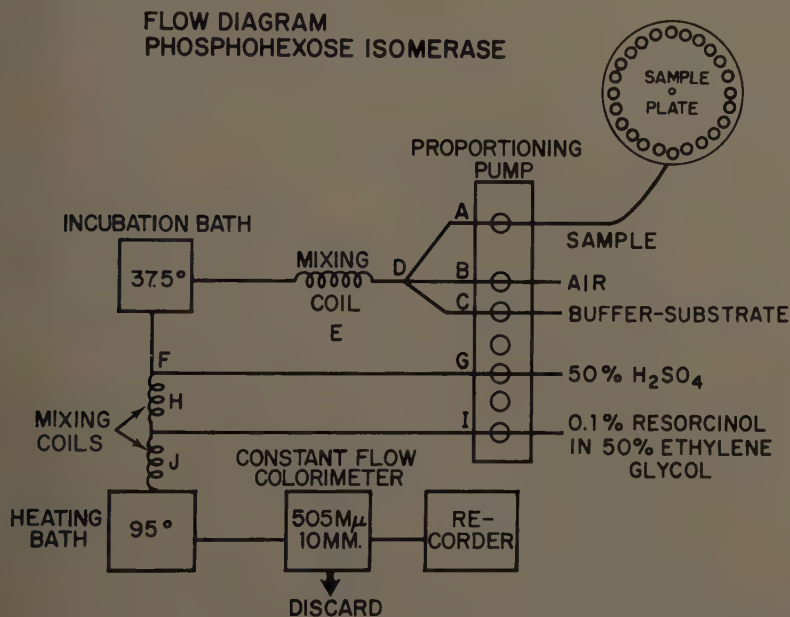


FIGURE 1. Flow diagram for the automated determination of PHI activity. See text for further details.

*The Automated Method for PHI Activity; Development of the Manifold  
for the Substrate-Enzyme Mixture*

The reaction mixture for the manual method contained the following concentrations of reactants: 0.002M G-6-P, 0.057M Veronal-acetate buffer at pH 7.4, and 0.20 ml. of enzyme preparation per ml. of reaction mixture. Accordingly, a glass-plastic manifold was developed (FIGURE 1) that delivered the reagents at rates yielding a reaction mixture of a composition as close as feasible to that in the manual procedure. The enzyme sample, diluted 1:5 or 1:10 in the case of serum and usually 1:1000 or 1:2000 homogenate in the case of tissue, is introduced through a plastic tube, A, delivering 0.32 ml./min. A substrate-buffer mixture or, in the case of blanks, buffer only is introduced through C at a rate of 1.20 ml./min. Since the acetate-Veronal buffer used in the manual method was found to precipitate and clog the system in the

course of color development, it was replaced by 0.1M tris(hydroxymethyl-aminomethane) buffer at pH 7.4. This substitution was not found to affect PHI activity as determined manually in several experiments. The delivery of the substrate mixture yielded a final enzyme reaction mixture with a G-6-P concentration of  $\frac{1.20 \times 0.0025}{1.20 + 0.32}$  or 0.00197M, a buffer concentration of  $\frac{1.20 \times 0.10}{1.20 + 0.32}$  or 0.08M, and an enzyme preparation concentration of  $\frac{0.32}{1.52}$  or 0.21 ml. per milliliter of reaction mixture. If a serum was diluted 1:5 before aspiration, the reaction mixture contained 0.042 ml. per reaction mixture. Similarly, a 1:1000 tissue homogenate would yield a final concentration of 0.211 mg. per milliliter of reaction mixture. The reaction mixture was segmented by air coming through tube B at a rate of 1.20 ml. per minute.

#### *Incubation of the Substrate-Enzyme Mixture*

The enzyme reaction mixture is mixed by gravity in its passage through a glass helix (F) with 13 turns, and is then introduced into a 37.5° C. incubation bath. The bath contains a 40-foot coiled glass tubing immersed in diethylene glycol maintained at 37.5° C. with a heating element and a mercury thermostat. The time of incubation, a function of the flow rate, was about 11 min. in the case of the PHI determination. This time is measured from the point where the substrate and sample join at point D to the point F, where reagent is added to stop the enzyme reaction. The incubation time changes slightly from day to day, probably due to change in temperature and in tubing diameter as the tubing ages and is stretched. The incubation time is easily measured each day by following the course of a colored solution (4 per cent potassium ferricyanide) and determining the exact time required for transit between D and F. In its transit from point D to the incubation bath, the enzyme reaction mixture is at 25 to 30° C. for about 30 sec. It can be shown, both theoretically and experimentally, that the lowering in reaction velocity due to this factor is negligible.

#### *Stopping of the Enzyme Reaction*

In many enzyme reactions, trichloroacetic acid is used to stop the reaction at a stated time and simultaneously to precipitate protein so as to avoid possible interference with subsequent colorimetric determinations on aliquots of the filtrate. In present automated procedures there is no provision for continuous centrifuging or filtering of precipitates. The component to be analyzed is separated from the protein by controlled dialysis. However, in the case of enzyme determinations such a procedure might introduce variable conditions unless the enzyme reaction was stopped before the products were removed by dialysis.

In the manual method for serum PHI activity, 2.5 ml. of 10 per cent trichloroacetic acid is added to an equal volume of reaction mixture at the end of 30 min.<sup>4</sup> After the precipitated proteins are filtered off, 6 ml. of 30 per cent hydrochloric acid is added to a 2 ml. aliquot of the filtrate which contains 1.0 ml. of the original reaction mixture. To this mixture is added 2 ml. of 0.1

per cent resorcinol in ethyl alcohol for color development. TABLE 1 shows that when 1 ml. of water and 6 ml. of 30 per cent hydrochloric acid were added directly to 1 ml. of reaction mixture, followed by heating with a 0.1 per cent solution of resorcinol in alcohol, the result was the same, within experimental error, as that obtained upon the analysis of equivalent aliquots of the trichloroacetic acid filtrate.

The introduction of 30 per cent hydrochloric acid at G to stop the reaction at F was found to clog the polyvinyl chloride tubing from G to F and at points subsequent to F after a flow of several hours. Examination of the inner surface of the tubing showed an interaction between the inner surface of the tubing and the 30 per cent hydrochloric acid, causing hardening and flaking of the tubing.

In addition to stopping the reaction, the function of the hydrochloric acid in the analytical procedure is to dehydrate the fructose moiety to hydroxy-

TABLE 1  
INFLUENCE OF PROTEIN ON ANALYSES FOR SERUM  
PHOSPHOHEXOSE ISOMERASE ACTIVITY

Serum specimen	F-6-P formed in 30 min. per ml. of reaction mixture, as determined on aliquot		Difference (μg.)
	Protein-free filtrate (μg./ml.)	Reaction mixture (μg./ml.)	
1	47	43	4
2	25	22	3
3	46	42	4
4	28	28	0
5	51	51	0

methylfurfural so that the latter may interact with resorcinol to form a colored compound.<sup>9</sup> Consequently, the effect of other acids was explored. Concentrated nitric, phosphoric, or glacial acetic acids, diluted 1:1 with water, failed to produce any color. In contrast, sulfuric acid interacted with fructose or F-6-P and resorcinol to give a cherry red color, as previously reported,<sup>9</sup> but did not react with the tubing to produce clogging. A substantial intensity of color was obtained with 50 per cent sulfuric acid, although at any given concentration of fructose or F-6-P high concentrations of sulfuric acid up to 100 per cent gave increasing optical density. When 1 ml. of water and 6 ml. of sulfuric acid, ranging from 50 to 70 per cent, were added to 1 ml. of reaction mixture, serum protein was not precipitated. The color obtained on interaction of fructose or F-6-P with resorcinol with 50 per cent sulfuric acid had the same spectrum as that with 30 per cent hydrochloric acid.

Since serum interacts with acid and resorcinol to give a red color, the effect of increasing concentrations of sulfuric acid on this "blank" value in the determination of PHI activity was explored. With water instead of serum running through tube A, air through tube B, buffer through tube C, and 0.1 per cent resorcinol in ethanol through tube I, sulfuric acid was introduced through



tube G. A slight color developed and was reflected in a recorded optical density of about 0.004. The recorder was now adjusted to give a base-line optical density of 0.000. Serum was now substituted for water in tube A. In a typical run, the resulting optical densities were: 0.105 with 50 per cent sulfuric acid, 0.224 with 60 per cent sulfuric, and 0.409 with 70 per cent sulfuric acid. These values, coming after adjustment of base line, represent the blank values or, in other words, the optical densities in the absence of any action or enzyme

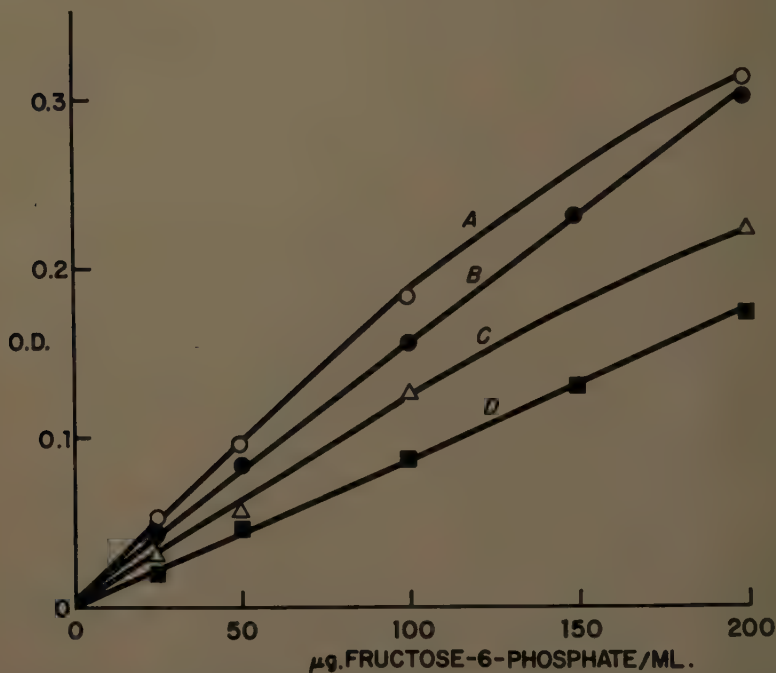


FIGURE 2. Optical density of the colored compound obtained on interaction of F-6-P with resorcinol and acid. A: 0.1 per cent resorcinol in ethanol and 30 per cent hydrochloric acid in a cuvette with a 6-mm. light path; B: 0.1 per cent resorcinol in 50 per cent ethylene glycol and 50 per cent sulfuric acid in a cuvette with a 10-mm. light path; C: 0.1 per cent resorcinol in ethanol and 50 per cent sulfuric acid in a cuvette with a 6-mm. light path; D: 0.1 per cent resorcinol in 50 per cent ethylene glycol and 50 per cent sulfuric acid in a cuvette with a 6-mm. light path.

on substrate. Because of the relatively large blanks at the higher concentrations of sulfuric acid, it was decided to use the 50 per cent concentration in the final analysis. Curves A and C in FIGURE 2 show that the optical densities obtained at various concentrations of F-6-P with this concentration of sulfuric acid and 0.1 per cent resorcinol in ethanol are about 70 per cent of those obtained with 30 per cent hydrochloric acid and the resorcinol-ethanol mixture.

#### *Substitution of Ethylene Glycol for Ethanol*

In the manual method and the preceding studies, resorcinol was dissolved in ethanol. However, when pumped through the automation apparatus and

heated during the color development stage, the ethanol begins to boil (b.p. 78.5° C.). The vapor pressure and formed vapor of the boiling ethanol occasionally disrupted the smooth flow of the colored mixture, resulting in noisy recordings and even periodic and abrupt blockage of solution flow. Ethylene glycol was found to be a good solvent for the resorcinol; because of its high boiling point (197.5° C.), the difficulties that were seen with the ethanol were overcome. However, the color intensity was decreased. As may be seen in FIGURE 2*d*, the optical densities of standards obtained with 50 per cent sulfuric acid and 0.1 per cent resorcinol in ethylene glycol were about one half of those obtained with 30 per cent hydrochloric acid and 0.1 per cent resorcinol in ethyl alcohol (FIGURE 2*a*).

A cuvette with a 10-mm. optical path was substituted for the 6-mm. cuvette in order to increase the optical densities. The resulting values were practically

TABLE 2  
EFFECT OF COLOR DEVELOPMENT HEATING TIME ON DETERMINATIONS OF  
PHOSPHOHEXOSE ISOMERASE OF HUMAN LIVER

Concentration in reaction mixture (mg. tissue/ml.)	Time (sec.)	Change in optical density	F-6-P formed (μg./ml.)	PHI activity (kilounits)*
0.840	100	0.125	144	18.3
	200	0.155	136	17.3
0.420	100	0.080	65	18.8
	200	0.100	69	17.5
0.210	100	0.046	34	17.3
	200	0.058	35	17.8
0.105	100	0.026	17	17.3
	200	0.032	16	16.3
Standard (100 μg.)	100	0.119	—	—
	200	0.160	—	—

\* See TABLE 3.

equal to those obtained with 30 per cent hydrochloric acid and resorcinol in ethanol with the 6-mm. cuvette (FIGURE 2*b*).

#### *Heating for Color Development*

As may be seen from FIGURE 1, the formed F-6-P, acid, and resorcinol solution were mixed in helices H and J and then passed into a heating bath at 95° C. The time, 1 min. and 40 sec., during which the mixture remained at this temperature was dependent on the flow rate and the length of coil in this bath. The question arose as to how the intensity of the color developed under these conditions compared with that developed by heating F-6-P with the usual mixture of 30 per cent hydrochloric acid and the alcoholic solution of resorcinol at 80° C. for 15 min.<sup>4,5</sup> The heating of fructose or F-6-P under the conditions of the automated method at 95° C. yielded a maximum color intensity in 5 min.; 80 per cent of this maximum was attained after 1.5 min. (90 sec.) of heating. In TABLE 2, column 3, are compared the optical densities obtained

by passage through one heating bath (100 sec.) and those obtained by passage through two heating baths joined in series. Although the latter optical densities were higher, comparisons with the calibration curves run at these two intervals yielded essentially the same values for the amount of F-6-P formed (column 4). Column 5 shows the activities in terms of the units to be described below.

### *Colorimeter*

In the manual method previously described for the determination of PHI activity,<sup>4</sup> the optical density of the cherry red-colored product formed by the interaction of the formed F-6-P with hydrochloric acid and resorcinol was determined at 490  $m\mu$  in a Coleman Junior Spectrophotometer. Comparison in the Beckman DK-2 recording spectrophotometer of this color with the one used in the automated method and formed by the interaction of F-6-P with sulfuric acid and resorcinol in ethylene glycol showed similar absorptions with a peak transmission between 490 and 495  $m\mu$  (H. B. Bachrach, personal communication). Since a 505- $m\mu$  filter was available for the automated colorimeter, this was used in the present method. The transmission at 505  $m\mu$  is 92 per cent of the peak transmission between 490 and 495  $m\mu$ .

### *Recorder and Calculations*

An equation was previously proposed by means of which enzyme activity may be expressed in terms of the amount of substrate changed at any stage within or beyond the zero-order portion of the reaction.<sup>6</sup> This formulation permits the use of low initial concentrations of substrate, a marked advantage in the case of automated procedures where large volumes of solutions of substrate are used as a result of the continuous flow.

In the case of PHI,<sup>4</sup> this equation is:

$$\text{Activity of unknown enzyme} = \frac{1}{E_a'} = \frac{E_b}{E_a E_b'} \quad (1)$$

where  $E_a$  is the concentration of a reference preparation of PHI that, under the standard conditions described, produced 25  $\mu\text{g.}$  fructose as F-6-P in 30 min.  $E_b'$  is the concentration of PHI that forms a measured amount of F-6-P in 30 min., and  $E_b$  is the amount of reference enzyme that would produce this same measured change in 30 min. Tables have been prepared that give directly the value of the enzyme activity ( $1/E_a'$ ) from the experimentally determined change in substrate.<sup>4</sup>

In the automated method, the reaction time  $t$  is usually from 10 to 11 min. instead of 30 min. It has been shown previously that in the case of PHI preparations the reciprocal of the time necessary to achieve a stated change in the substrate is directly proportional to the concentration of enzyme present. It may be shown that for a time  $t$  other than 30 min. EQUATION 1 has the following form:

$$\text{Activity of unknown enzyme} = \frac{1}{E_a'} = \frac{E_b}{E_a E_b' (t/30)} \quad (2)$$



In other words, the amount of F-6-P formed per milliliter of reaction mixture in the automated method in  $t$  minutes is translated into units of enzyme activity by means of EQUATION 1 multiplied by  $(30/t)$ . To facilitate expression of isomerase activities, TABLE 3, previously constructed by means of EQUATION 1 for serum PHI, is presented to include expression of tissue PHI activity.

The conversions of  $\mu\text{g.}$  fructose as F-6-P units of PHI activity listed in TABLE 3 hold for a concentration of 0.04 ml. serum or 0.200 mg. of tissue per milliliter of reaction mixture. If concentrations other than these are used, as in the

TABLE 3

TABLE FOR CONVERTING AMOUNTS OF FRUCTOSE FORMED AS FRUCTOSE-6-PHOSPHATE PER CU. CM. UNDER STANDARD REACTION CONDITIONS INTO UNITS OF PHOSPHOHEXOSE ISOMERASE ACTIVITY\*

$\mu\text{g.}$	Activ- ity units	$\mu\text{g.}$	Activ- ity units	$\mu\text{g.}$	Activ- ity units	$\mu\text{g.}$	Activ- ity units	$\mu\text{g.}$	Activ- ity units	$\mu\text{g.}$	Activ- ity units	$\mu\text{g.}$	Activ- ity units
1	1	21	21	41	41	61	67	81	101	101	147	121	212
2	2	22	22	42	42	62	69	82	103	102	149	122	216
3	3	23	23	43	43	63	70	83	106	103	153	123	220
4	4	24	24	44	44	64	72	84	107	104	155	124	224
5	5	25	25	45	45	65	74	85	109	105	158	125	229
6	6	26	26	46	46	66	76	86	111	106	161	126	234
7	7	27	27	47	47	67	78	87	113	107	163	127	240
8	8	28	28	48	48	68	80	88	115	108	166	128	245
9	9	29	29	49	50	69	81	89	117	109	170	129	252
10	10	30	30	50	51	70	84	90	120	110	172	130	257
11	11	31	31	51	52	71	84	91	122	111	176	131	264
12	12	32	32	52	54	72	86	92	123	112	179	132	272
13	13	33	33	53	55	73	88	93	126	113	182	133	278
14	14	34	34	54	56	74	90	94	128	114	186	134	286
15	15	35	35	55	58	75	92	95	131	115	190	135	293
16	16	36	36	56	60	76	93	96	133	116	194	136	300
17	17	37	37	57	61	77	94	97	136	117	198	137	307
18	18	38	38	58	62	78	97	98	139	118	202	138	314
19	19	39	39	59	64	79	99	99	141	119	206	139	324
20	20	40	40	60	66	80	100	100	144	120	208	140	331

\* This table applies at concentrations of 0.04 ml. of serum or 0.200 mg. of tissue per ml. of reaction mixture. When the concentration of serum or tissue in the plastic cups on the turntable is such as to yield  $n$  times these concentrations in the reaction mixture, the units should be multiplied by  $1/n$ .

automated procedure with a concentration of 0.042 ml. serum or 0.210 mg. tissue, the conversion factors are the same for the zero-order portion of the curve, that is, up to about 49  $\mu\text{g.}$  fructose formed as F-6-P. This relationship holds since, in the automated procedure, the concentration of standard is increased to the same extent as the concentration of enzyme; the amount of F-6-P formed during the zero-order portion is directly proportional to the concentration of enzyme.

Beyond the zero-order portion of the curve, the amount of F-6-P formed at a concentration of 0.042 ml. of serum or 0.210 mg. of tissue is less than called for by direct proportionality. In contrast, the optical density of a solution containing 0.042 ml. of various standards is directly proportional to that of a solution containing 0.040 ml. Hence a factor is necessary to correct the

amounts of F-6-P obtained with 0.042 ml. of serum before conversion is made according to TABLE 3. At this concentration of serum, the factor is 1.01.

FIGURE 3 shows a recording of the automated analyses of a series of standards

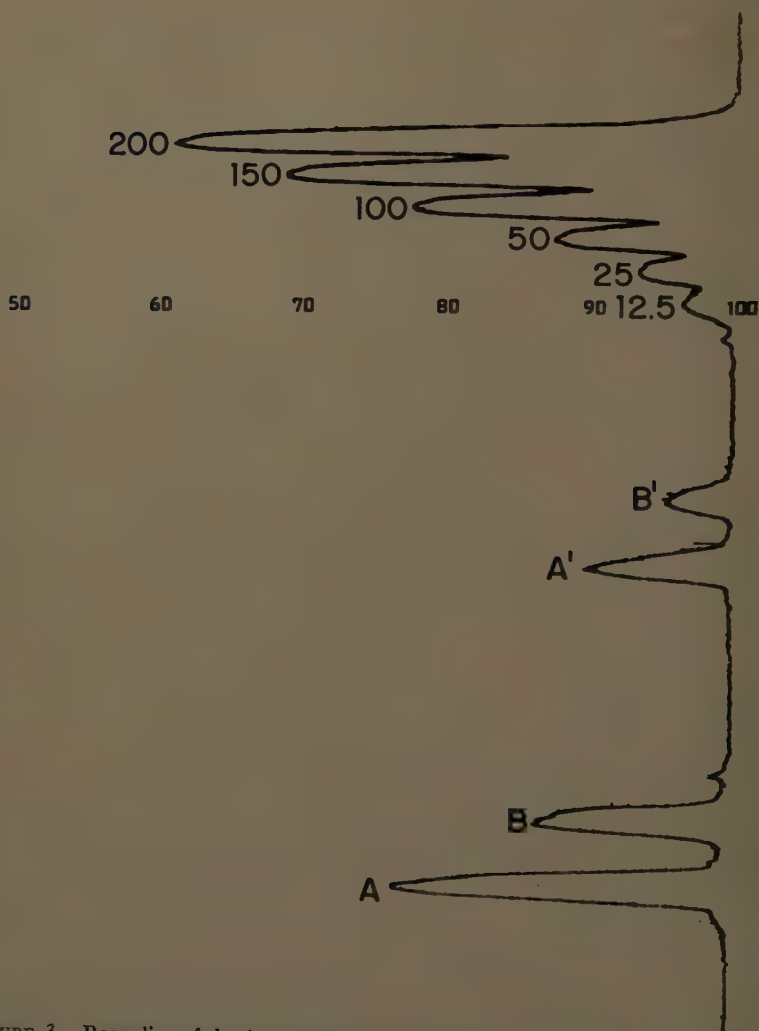


FIGURE 3. Recording of the determination of the PHI activity of a serum. Concentration at A, 0.042 ml. per milliliter of reaction mixture; at B, 0.021 ml. per milliliter of reaction mixture. A' and B' represent the corresponding blanks. The standards ranged from 12.5  $\mu$ g. to 200  $\mu$ g. per milliliter of reaction mixture. See text for details of calculation.

and of PHI activity of a serum determined at concentrations of 0.042 and 0.021 ml. of serum per ml. of reaction mixture. These readings were 65  $\mu$ g. and 39  $\mu$ g., respectively. Consequently, 65  $\mu$ g. was multiplied by 1.01 to yield 66  $\mu$ g., whereas no correction was necessary for the value of 39  $\mu$ g. Reference to TABLE 3 showed that these values corresponded to 76 and 78 units, respectively,

or an average of 77 units of isomerase activity for an incubation period of 10.92 min. Accordingly,  $77(30/10.92)$  or 212 units was the actual isomerase activity.

In the present automated method, the tubing aspirates a serum dilution or a homogenate to yield a final concentration of, respectively, 0.042 ml. of serum or 0.210 mg. tissue per milliliter of reaction mixture. Since the inner diameter of the tube may vary from the manufacturer's specifications or may change with use, the final concentration of enzyme may differ from the stated values. In accordance with EQUATION 1, the micrograms of F-6-P formed were calculated at various concentrations of enzymes from the corresponding calibration curves. It was found that, for the major part of the relationship beyond the

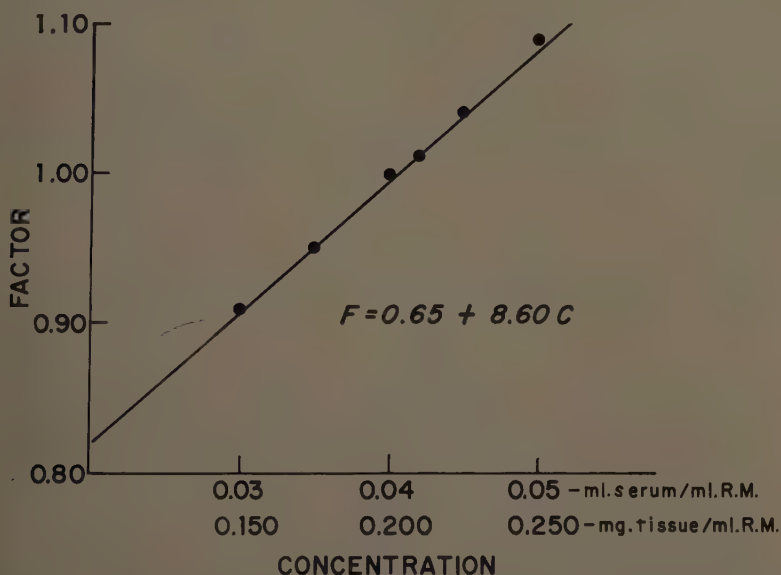


FIGURE 4. Relationship between concentration of enzyme and correction factor for use when the amount of F-6-P formed is beyond the zero-order portion of the reaction and when variation in the concentration of enzyme or standard is due to the changing diameter of tubing in the manifold.

zero-order portion represented by EQUATION 1, a factor converted the F-6-P formed at one concentration of enzyme to that at another. These factors were calculated for several concentrations of enzyme as shown in FIGURE 4 and were found to fit a linear regression equation,  $F = 0.65 + 8.60 c$ , where  $c$  is the concentration of enzyme in the reaction mixture and  $F$  is the factor by which the amount of F-6-P formed is multiplied before conversion to units in accordance with TABLE 3. The concentration of enzyme preparation is determined, as has been noted earlier in this paper, from its flow rate and the flow rate of the substrate-buffer mixture. These rates may in turn be determined quite simply on any day's run by measuring the amounts of water drawn by tubes A and C (FIGURE 1) from separate graduates for a measured period of time.



*Comparison of PHI Activities Obtained by Automated and Manual Methods*

The PHI activities of each of five sera were determined on each of three days by the automated and the manual procedures (TABLE 4). The results obtained by these two methods did not differ significantly from each other. Similar results for rat and human liver are shown in TABLE 5. In additional experiments, it was found that tissue homogenates kept in plastic cups at room temperature for as long as 1 hour, the time for a complete revolution of the sample plate, underwent no change in activity.

TABLE 4  
COMPARISON OF MANUAL AND AUTOMATED METHODS IN DETERMINATION OF  
PHOSPHOHEXOSE ISOMERASE ACTIVITY OF SERUM

Serum specimen	Manual		Automated	
	Mean value (units)	Average deviation (units)	Mean value (units)	Average deviation (units)
1	35	1	35	2
2	39	2	38	2
3	95	3	97	3
4	71	2	72	6
5	78	7	76	1

TABLE 5  
COMPARISON OF LIVER HOMOGENATE PHI ACTIVITY DETERMINED MANUALLY  
AND BY AUTOMATED TECHNIQUE  
(Values Represent Averages at 2 Different Dilutions of Homogenate)

Tissue	Manual (kilounits/gm.)	Automated (kilounits/gm.)
Rat liver	7.35 $\pm$ 0.75	6.9 $\pm$ 0.0
Human liver 1	33.0 $\pm$ 2.9	33.5 $\pm$ 1.5
Human liver 2	28.4 $\pm$ 0.2	22.7 $\pm$ 0.7

*Automated Analyses of PHI Activities of Brain, Liver,  
Spleen, and Kidney of the Mouse*

The advantage that an automated method might have in experiments requiring many enzyme analyses was determined by the following time study on the determinations of PHI activity of 4 organs in 10 mice. At 9:20 A.M. the first animal was sacrificed; the kidney, spleen, liver, and brain were removed, weighed, and homogenized with water to a 1:100 dilution in a Potter-Elvehjem tube in the cold room. Two further dilutions, 1:1000 and 1:2000, were made from the 1:100 homogenate. These represent tissue concentrations of 0.211 and 0.106 mg. of tissue per ml. of reaction mixture, respectively. By 10:00 A.M. these 8 homogenates from one animal were placed in plastic cups on the turntable, and their analyses begun. The adjustment of the apparatus, the analysis of standard F-6-P solutions, the determination of the incubation time, and the preparation of the record book had been accomplished in the preceding

40 min. At 10:10, the second mouse was sacrificed, and thereafter, except during the lunch period, further sacrifices occurred at intervals of approximately 20 to 30 min. The cooperation of two persons in the weighing of the organs, the homogenizations, and the dilutions permitted a fairly smooth and continuous delivery of samples to the turntable. The tenth and last animal was sacrificed at 2:30 P.M. Thereafter, only one person was involved in the determinations, and the last value appeared on the recorder at 4:00 P.M. Calculations from the recorder and standard curve of the F-6-P formed and final conversion of these values to units of PHI activity required another hour and one half. Thus the entire experiment, consisting of the removal, preparation, and analyses of 40 tissues, each at two dilutions, was completed in about 12 man-hours of working time. Comparison with previous manual determinations in this laboratory indicated that the speed of performance with the automated procedure was about eight times as great.

The values for the determination of PHI in each tissue at the two concentrations, 0.211 and 0.106 mg. of tissue per ml. reaction mixture, were averaged. Of the 40 average values, slightly over 70 per cent had average deviations of

TABLE 6  
MEAN PHOSPHOHEXOSE ISOMERASE ACTIVITY OF TISSUES IN TEN MICE

Tissue	Mean value (kilounits)	Standard deviation (kilounits)
Liver	24.1	2.5
Kidney	37.2	2.9
Brain	39.4	6.9
Heart	33.4	2.6

less than 5 per cent. Three of the values had deviations between 10 and 20 per cent, and nine had deviations between 0 and 1 per cent. The mean values for the activities of each tissue in 10 mice are shown in TABLE 6. The calculation of tissue activity may be illustrated briefly in accordance with the definitions previously given in this paper. In one analysis, a concentration of 0.211 mg. per milliliter of reaction mixture actually formed  $\frac{0.211}{0.200} \times 47 \mu\text{g.}$  fructose as F-6-P in 11 min., 59 sec., as read from the calibration curve and in accordance with the consideration previously presented. Since the change is on the zero-order portion of the curve, it is equivalent to the formation of 47  $\mu\text{g.}$  by 0.200 mg. of tissue per milliliter of reaction mixture. In accordance with EQUATION 2, this value represents 118 units. Therefore, 25 units would have been given by a concentration of  $\frac{25}{118} \times 0.200$  or 0.0424 mg. The reciprocal of this is 23,600 units, or 23.6 kilounits.

It is of interest to note that the automated method that has been described may be applied to nonenzymic analyses involving the determination of fructose or fructose derivatives. Thus it has been found in this laboratory that inulin is hydrolyzed completely under the conditions of the automated method,

and the rapid determination of this component in renal clearance and extra-cellular volume studies seems quite feasible.

### *Summary*

The chief features in the adaptation of a manual enzyme method to an automated procedure as demonstrated by the present work are (1) the choice of tubing to yield a flow reaction mixture as close as possible in composition to that of the manual procedure for which the reaction conditions have been defined; (2) substitution of reagents used in the manual method that react with the plastic tubing or interfere with the smooth flow as the result of precipitation, generation of excessive vapor pressure, or some similar phenomenon; (3) stopping the reaction at a measured time without the precipitation of protein; and (4) the use of proper measures of reaction velocity in determining enzyme activities, particularly in connection with changes in flow rates and, consequently, in enzyme concentration.

This study shows that the automated method for PHI is approximately eight times as rapid as the manual procedure, yet it retains the same precision and reproducibility.

### ACKNOWLEDGMENT

We gratefully acknowledge the excellent technical assistance of Anita Smith.

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# CHOLINESTERASE ACTIVITY DETERMINATION IN AN AUTOMATED ANALYSIS SYSTEM\*

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The determination of the esteratic activity of acetylcholinesterase (ChE) has been used as a primary or secondary diagnostic aid in diverse areas of medicine. In phosphate ester insecticide or nerve (war) gas poisoning, the inhibition of ChE is said to be the principal effect;<sup>7,8,20</sup> the estimation of ChE depression therefore becomes extremely important. For the same reason, the determination of ChE activity is a necessary part of the toxicological evaluation of those insecticides and compounds that are ChE inhibitors. The determination of serum ChE activity has been used in assessments of hepatic function as one index of hepatocellular damage.<sup>10,13,17,18</sup> Erythrocyte ChE has been used to indicate the rate of manufacture and the longevity of red cells in the body; it is a very useful aid in the differentiation of certain types of anemias, while providing a valuable clue to bone marrow condition and function.<sup>2,21</sup>

The most commonly accepted methods for the determination of ChE are time-consuming and require a relatively high degree of operator precision and training in the use of expensive or unusual equipment. It is felt that these are among the more important factors limiting the use of the ChE test.

Numerous investigators have presented new methods or modifications calculated to improve or simplify the determination of ChE activity. Most of these tests depend either on the rate of formation<sup>23</sup> or the amount of acetic acid liberated from the hydrolysis of acetylcholine by the enzyme. The acid formed is measured electrometrically,<sup>6,16,24</sup> colorimetrically, or manometrically by the measurement of the carbon dioxide liberated from bicarbonate buffer by the acid.<sup>1</sup> Colorimetric methods include the direct hydrolysis of indophenyl acetate to form a highly colored compound<sup>12</sup> and indirect methods such as the formation of acethydroxamic acid and thence colored ferric acethydroxamate from the unreacted acetylcholine<sup>9</sup> and colorimetric estimation of the SH groups in thiocholine (from the enzymatic splitting of acetylthiocholine) by the sodium nitroprusside reaction.<sup>15</sup> Other variations include the bromothymol blue indicator<sup>13,14</sup> and ultraviolet absorption<sup>10</sup> methods.

The development of an automated continuous flow system of wet-chemical analysis<sup>22</sup> has made it possible to adapt the cholinesterase determination to an automatic assay technique. Acetic acid liberated by the hydrolysis of acetylcholine in a buffered system (after Michel<sup>16</sup>) is estimated colorimetrically as the color change of phenol red indicating the change in  $pH$ .<sup>4</sup>

The procedure as detailed below offers enormous savings in personnel and time over conventional methods. It is not only accurate and reproducible, but also versatile, as shown by successful use in determinations of ChE activity in whole blood, plasma, and erythrocytes of humans, dogs, and rats; brain homogenates of rats and brei of housefly heads.

\* The experiments initiating the development of this method were performed in the Environmental Health Laboratories of the American Cyanamid Co., Stamford, Conn.



*Apparatus*

The laboratory model of the AutoAnalyzer\* consists of a series of separate modules interconnected by polyvinyl chloride tubing, through which the samples and the various reagents are propelled by a peristaltic-type pump. Its components are as follows:

(1) A circular sampler plate carrying separate samples. This is driven by a synchronous motor in such a manner that each sample is presented to a pick-up arm for a preset interval. The sampling period is followed by a short aspiration of air through the sample line to separate the values obtained for successive samples on the recorder.

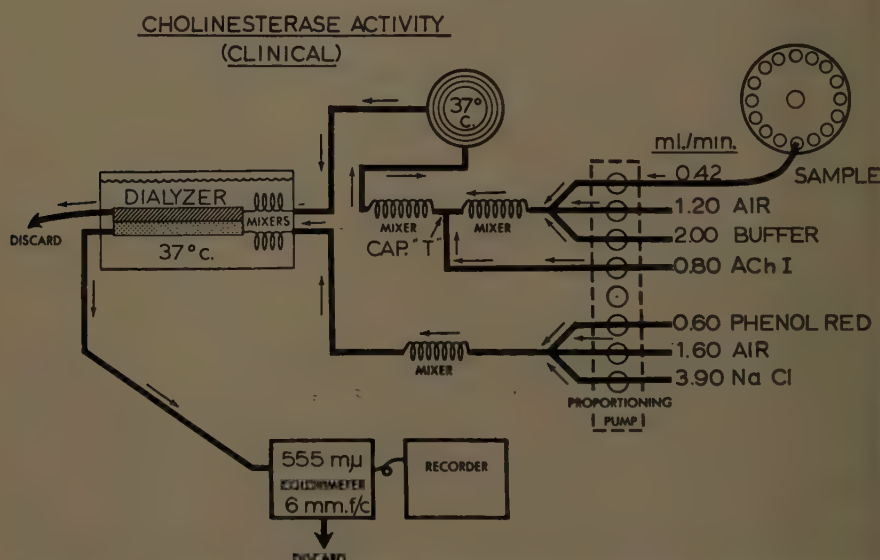


FIGURE 1. Diagram showing the flow paths and relative volumes of sample and reagent in the analysis system.

(2) A proportioning pump, consisting of a chain-driven set of rollers that pumps by continuously advancing a wave of compression that impels the liquid in a series of polyvinyl tubes. The volume of liquid propelled in each tube is dependent on the bore of the tubing used; thus different volumes of the various reagents may be introduced as required by the analysis. The sample is introduced to the reagent stream, then the reagent streams enter the system and are mixed in turn at appropriate stages in the analysis and impelled through the various modules, as shown in the accompanying flow diagram (FIGURE 1).

(3) A heating bath consisting of a length of glass tubing spirally wound and immersed in a thermostatically controlled, heated fluid.

(4) A dialyzer module composed essentially of two plates in which mirror-image grooves have been machined, clamped firmly on either side of and in contact with a semipermeable membrane. The whole is immersed in a con-

\* Manufactured by Technicon Instruments Corp., Chauncey, N. Y.

trolled-temperature water bath. As the reagent streams are impelled along the grooves, dialysis occurs across the membrane.

(5) A colorimeter-recorder, that is, a ratio-recording dual-beam, filter colorimeter having a continuous flow cuvette.

### *Materials*

The reagents were as follows:

Buffer I (Michell <sup>16</sup> )	
Sodium barbital	1.2370 gm.
Potassium phosphate (dihydrogen)	0.1360 gm.
Sodium chloride	17.535 gm.
Saponin*	0.100 gm.
Buffer II (Frawley <sup>5</sup> )	
Sodium barbital	0.4124 gm.
Potassium phosphate (dihydrogen)	0.0546 gm.
Sodium chloride	17.535 gm.
Saponin*	0.100 gm.

Each buffer mixture is dissolved in distilled water to make 1 l.

Buffer I is used for human plasma, serum, or erythrocytes, dog or rat brain homogenates, and fly head brei in concentrations of 10 or more heads per milliliter. Buffer II is more suitable, that is, it provides greater sensitivity, for dog and rat plasma and erythrocytes and fly heads in concentrations of less than 10 per milliliter. It is expected that buffer II would be required for determinations on the blood of such animals as rabbits and guinea pigs.

The substrate consists of 20 gm. acetylcholine iodide† dissolved in distilled water to make 1 l.

Phenol red WS‡ (0.225 gm.) is dissolved in distilled water to make 1 l. Sodium chloride (100.00 gm.) is dissolved and made up to 1 l. with distilled water.

The standard solution is 20,000 units per vial§ acetylcholinesterase. A stock solution is made by dissolving the contents of one vial in 10 ml. distilled water. Working standard solutions of 10, 20, 30, . . . , 100 units ChE per ml. are obtained by diluting 0.10, 0.20, 0.30 . . . , 1.00 ml., respectively, of stock solution up to 20 ml. each.

When buffer I is used, the effective range of the system will be encompassed by the standards up to 120 units, if desired. When buffer II is used, the 10-to-50 units/ml. standards will suffice. All solutions of ChE should be refrigerated when not in use.

### *Procedure*

A tubing manifold is prepared on the proportioning pump and the modules are arranged in sequence, according to the flow diagram (FIGURE 1). The color development with acetic acid formation is, in effect, inversely colorimetric,

\* Required only when testing whole blood or packed erythrocyte samples.

† Distillation Products Industries, Rochester, N. Y.

‡ Hartmann-Leddon Co., Philadelphia, Pa.

§ Units are those of the manufacturer, Winthrop Laboratories, New York, N. Y., as determined by the method of Ammon.<sup>1</sup>

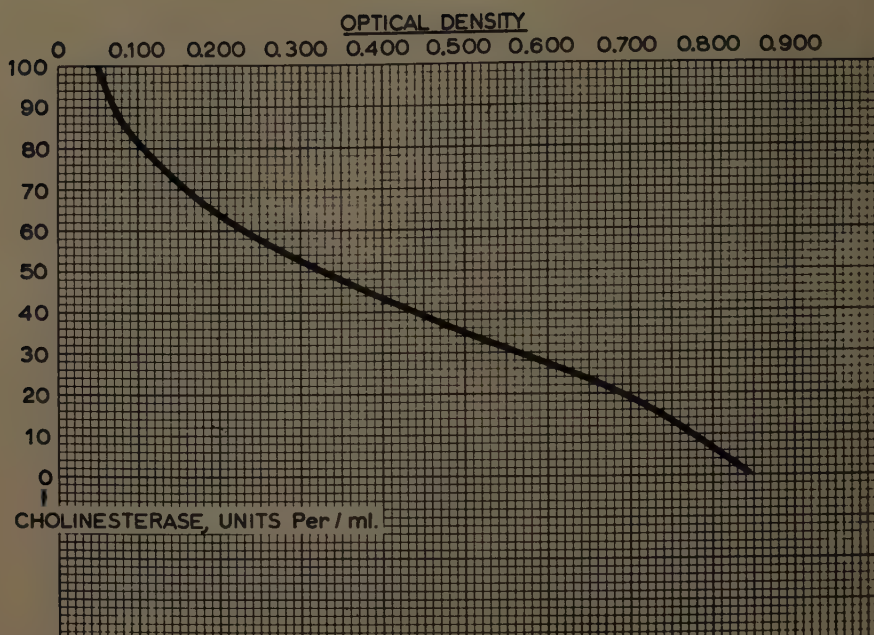


FIGURE 2. A typical calibration curve for ChE activity (buffer I).

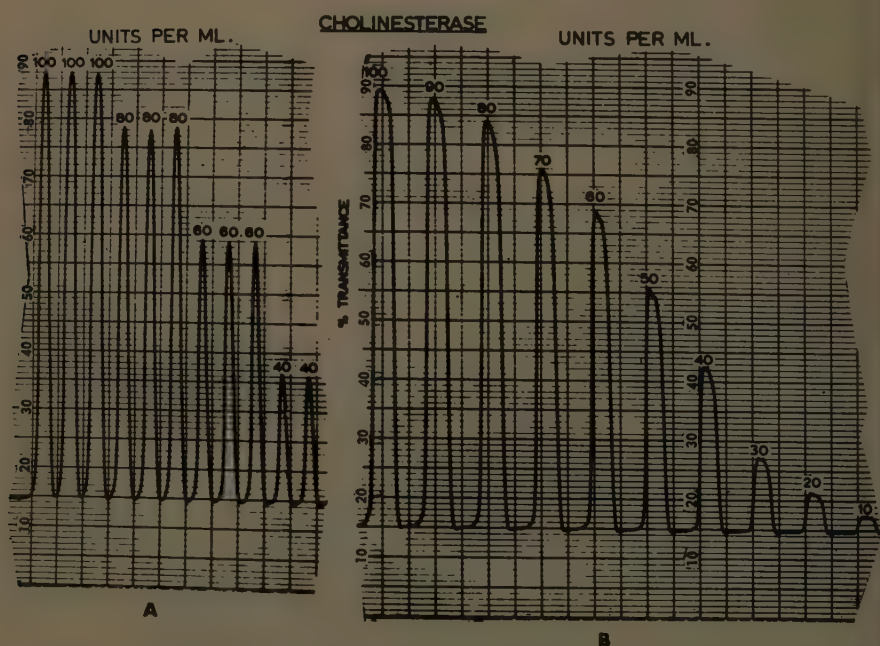


FIGURE 3. Recordings of a series of standard ChE solutions (buffer I). These were sampled at a rate of 40 per hour in A and 20 per hour in B with a water-wash sample cup following each standard.



that is, the absorbence of the alkaline (red) indicator decreases with the  $pH$  as the color becomes yellow. Accordingly, the instrument is adjusted to 100 per cent transmittance, with distilled water flowing through all the reagent and sample lines, which are then placed in their respective reagents to permit



FIGURE 4. A series of standard ChE solutions and human blood serum samples taken at 40 per hour (buffer I).

the plotting of a base line before the samples and standards are introduced. The recorder base line may be adjusted to the same level each day by preliminary adjustment of the  $pH$  of the buffer and of the NaCl recipient solution to 8.2 with a few drops of  $\frac{1}{10} N$  NaOH as required.

The sample cups are filled (without measuring) with samples and standards in the desired sequence, and the sampler is adjusted to run at 20 or 40 tests per hour, as required.

A calibration curve is obtained by making a semilog plot of per cent transmittance of the standards versus concentration. A typical standard curve is shown in FIGURE 2. Sample values (expressed as units of ChE per milliliter) are then determined by direct comparison with the standard curve.

### Discussion

A typical recording made with a series of standard samples is shown in FIGURE 3b. Sampling was done at the rate of 20 per hour, that is, 2 min. pick-

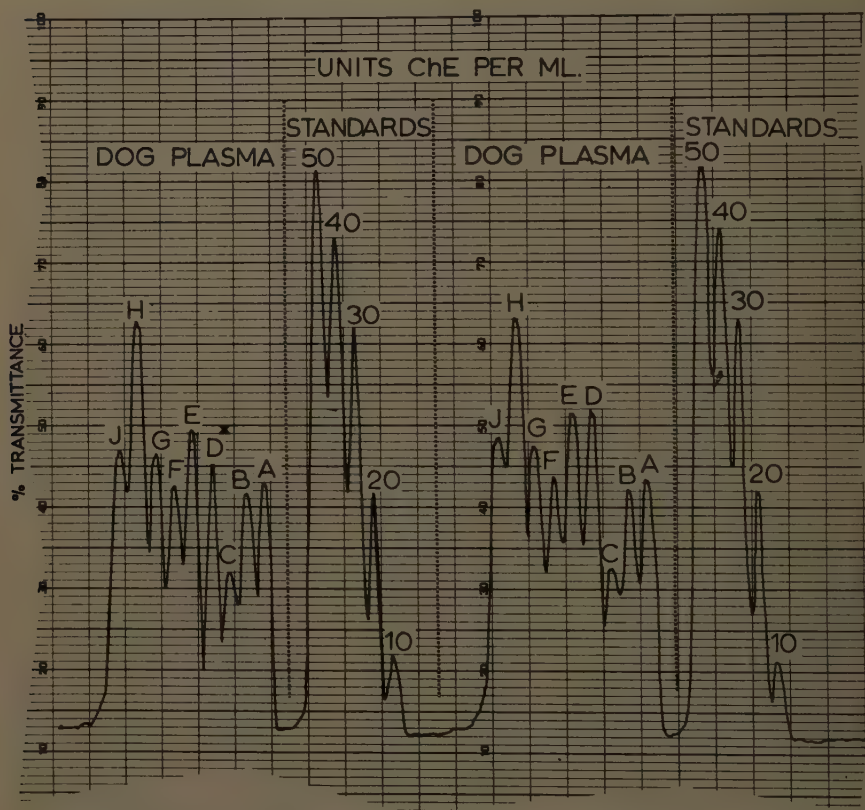


FIGURE 5. Duplicate sets of standard ChE solutions and dog plasma samples (buffer II). The sampling rate was 40 per hour, with no wash between samples. (Note that x, the duplicate of sample D, contained insufficient material for a full sample so that the apparent value does not agree with that of D.)

up or 0.84 ml. of each sample. The spacing between samples and the complete return to the base line was accomplished by placing a water-wash sample between the ChE samples. The triplicate samples in FIGURE 3a (taken at a sampling rate of 40 per hour with water wash between) are included to show the reproducibility of the system.

In FIGURE 4, a standard series is shown along with a random selection of pooled human serum samples. These sera were 4 to 6 months old when analyzed and had been frozen and thawed an unknown number of times. The change

in pH per hour of a mixture of these samples was determined as 0.57 by the electrometric method.<sup>24</sup> This is considerably lower than the normal values of 0.7 to 0.9 reported by several investigators,<sup>3,16,19,24</sup> and indicates a considerable loss in ChE activity of these samples.

Duplicate measurements of standards and fresh dog plasma samples are shown in FIGURE 5. The excellent agreement between the curves will be noted except in the case of plasma D, when the second sample contained insufficient material.

For some species, in which the ChE activity is normally low, such as rabbits, rats, and guinea pigs, it may be desirable or even necessary to increase the response per sample to obtain greater sensitivity. This may be done most readily with the use of larger than specified tubing for the sample line, or by increasing the time allowed for hydrolysis by means of an incubation coil of greater capacity. Other means for accomplishing this purpose include, of course, the use of a flow cuvette with a longer than specified light path or expansion of the electronic range of the recorder scale.

### Summary

A method for complete automation of cholinesterase activity determination is presented. Because continuous dialysis is a feature of the analytical system used, it is possible to use this method for determinations on various tissues from any animal or insect species. Typical recorder tracings are shown to demonstrate the fact that up to forty tests can be performed per hour with excellent precision and reproducibility.

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## CONTINUOUS AUTOMATIC INTEGRATED FLAME PHOTOMETRY

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Development of the analytical equipment discussed below was undertaken to fulfill the need in both industry and the clinical laboratory for continuous and repetitive automatic sodium and potassium analyses. Certain specialized equipment exists for industrial use,<sup>1</sup> but the present flame photometer has much broader utility. No suitable or comparable equipment for clinical use is known to us. When the flame photometer is combined with the AutoAnalyzer,\* continuous, automatic, and unattended analyses are possible.

The equipment represents a considerable advance over manual methods, which can accomplish only about ten serum K and Na determinations per hour of actual and elapsed time, including that required for making very large and accurate dilutions for each determination and running many standards of known concentration.

Development of techniques and instrumentation for sodium and potassium determinations, either separately or simultaneously from the same sample, has been favored, but other determinations are possible. Development continues for analysis of elements requiring higher excitation levels.

A general view of the equipment for sodium and potassium determinations is given in FIGURE 1. A quick review of the procedure and equipment will help to create the framework for more detailed subsequent discussions.

Successive samples are placed in the AutoAnalyzer sampler in the normal manner. In contrast to the manual requirement for careful dilutions, unmeasured samples are adequate. The proportioning pump delivers the sample, as well as other fluids, suitably and automatically proportioned to the dialyzer. The flow from one side of the dialyzer goes to waste, while the flow from the other is delivered to the burner in the flame photometer, which is fed propane and oxygen from the tanks and regulators shown at the extreme right. The flame photometer will be discussed in some detail subsequently. Output of the photometer is fed to the recorder. The record appears as sharply defined values for each sample. When used industrially for stream monitoring, the record will be continuous without periodic return to base line as seen with repetitive sampling. For determinations other than Na and K, other AutoAnalyzer modules might be required. With adequate quantities of sample and reagents supplied, the flame photometer system functions automatically and continuously without attention.

FIGURE 2 presents the system arrangement diagrammatically. The specific values indicated at the various points in the diagram are those presently used, but are not necessarily the final ones. The sampler, proportioning pump, and dialyzer are described adequately elsewhere in the monograph, and only brief remarks are necessary. The timed operation of the sampler assures that an adequate sample volume on a selected frequency basis (tests per

\* Technicon Instruments Corp., Chauncey, N. Y.

hour) is available to the pump. The proportioning pump meters sample, air, lithium nitrate ( $\text{LiNO}_3$ ) solution, and distilled water from bottles in the concentrations and volumes shown on the flow diagram. By appropriate connections two streams are created, one to each side of the dialyzer.



FIGURE 1. General view of flame photometer system.

$\text{Na}^+ \text{K}^+$  via FLAME

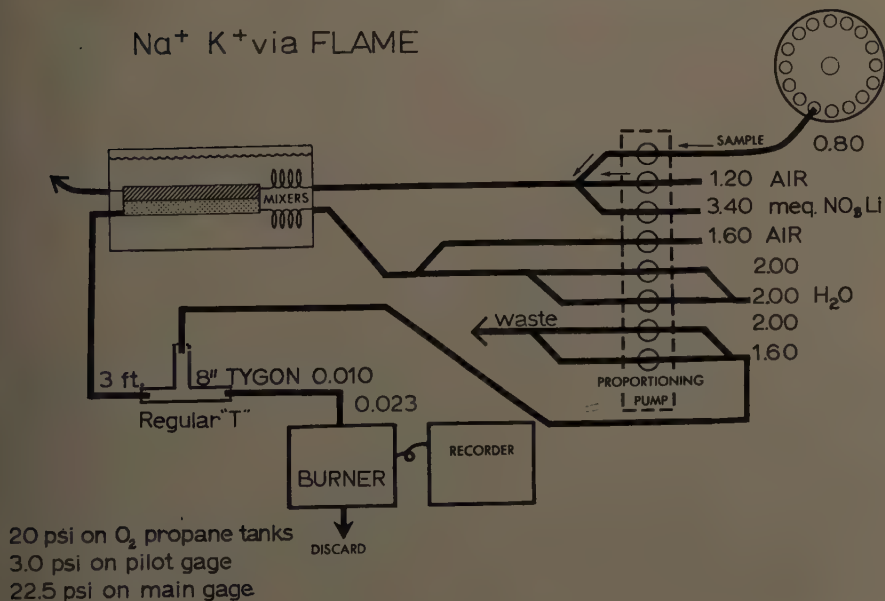


FIGURE 2. Flow diagram.

In manual flame photometric determinations of Na and K, careful predilutions of the specimen are required, and many errors can arise from lack of precision. In the automated system premeasured samples, standards, and diluents are not required. The pump dilutes accurately. The fluids pass through a glass helix that assures thorough mixing. Standards and samples are metered and diluted identically.

When Li is used as an internal standard, attention must be paid to control and proportion of numerous factors such as Li concentration.<sup>2,3</sup> With all factors optimized and maintained constant, Li provides excellent results in the present equipment.

In the present study 0.8 ml. of sample is diluted to a total volume of 4.2 ml. with  $\text{LiNO}_3$  at a concentration of 225 mEq./l. The diluted sample containing Li, which is used as an internal standard, enters the dialyzer where the Na, K, and Li diffuse into a recipient stream of distilled water pumped at a rate of 4 ml./min. For both the sample and recipient sides of the dialyzer, air is used for segmentation. As Li is present in the stream containing the sample, fluctuations in the dialyzing rate affect Na, K, and Li equally, which helps to cancel out any small fluctuation in dialyzing rates. The dialyzing stage also prevents the entry into the burner of unwanted material, including protein, since the pore size of the dialyzing membrane does not permit protein to diffuse across. The coarse particles found in industrial fluids are completely blocked. Essentially, the burner is supplied with an aqueous system containing the electrolytes as well as low molecular weight nonelectrolytes.

After dialysis the volume of the recipient stream containing Na, K, and Li is reduced before entering the burner, and the air used for segmentation is removed by feeding the stream into a T fitting. From the vertical side arm of the T, liquid and air are removed by the proportioning pump and discarded to waste. One ml./min. of liquid remains and is constantly pumped into the capillary of the burner under positive pressure. The vertical arrangement of the side arm assures that no air enters the burner.

This system of delivery to the burner provides a much more constant liquid flow rate than does aspiration. Partial clogging of the capillary, which changes aspirated flow markedly, has only slight effect on the pumped flow. The burner is nevertheless capable of aspiration, and this characteristic probably helps obtain good atomization and evaporation.

FIGURE 3 shows a typical recording plot of a standard curve for sodium, using fourfold amplification. The recorder chart moves at 18 in. per hour, with the sample determination at 40 per hour. The plot of the trace is made on linear graph paper. Approximately 75 divisions are obtained for 60 mEq. Na/l. running standards from 100 to 160 mEq. Na/l., giving a sensitivity of approximately 0.8 mEq. Na/l. per division. FIGURES 4a and 4b illustrate the reproducibility of standards at various levels. The reproducibility range is  $\pm 0.75$  mEq. Na/l. Recoveries of Na from a serum pool, run by diluting a pool and then adding sodium in 10 mEq./l. increments, show excellent results.

The determination of potassium in biological fluids is similar to that for



sodium. The same manifold and reagents are employed. The range for potassium is 2 to 8 mEq./l., which gives a sensitivity of approximately 0.1 mEq. K/l. per division. A standard curve at no amplification and its plot on linear graph paper are illustrated in FIGURE 5. The rate of sampling is 40 determinations per hour. FIGURE 6 shows replicate determinations of serum potassium with a reproducibility of  $\pm 0.15$  mEq. K/l. The addition of K to a serum pool gives excellent recoveries, as shown in FIGURE 7.

Performance of this precision requires considerable sophistication of the control scheme and reliability of components. In order to achieve high sen-

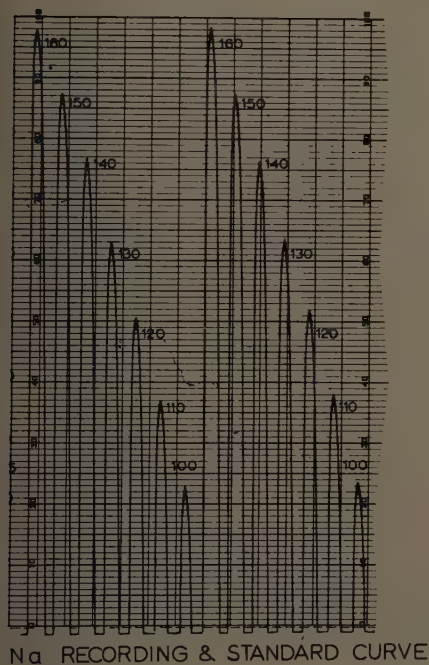
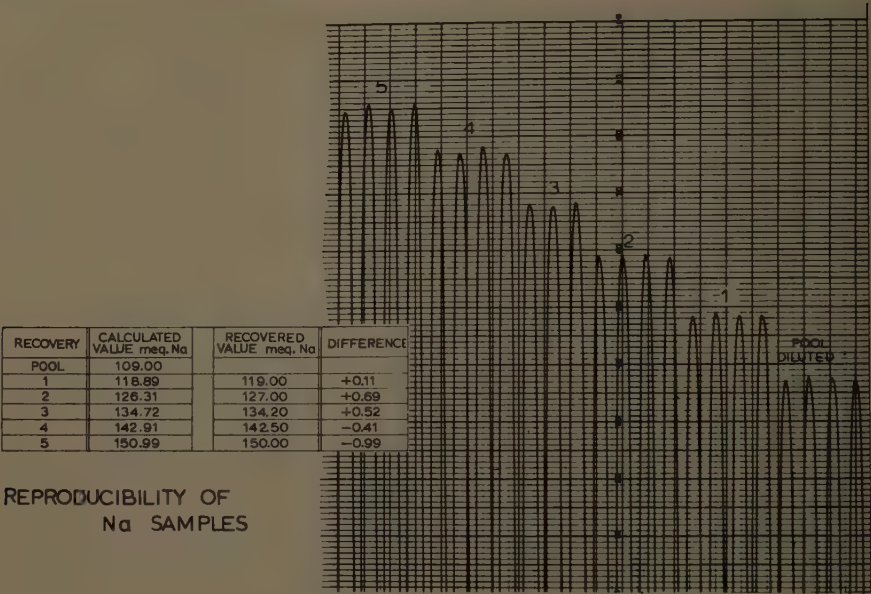


FIGURE 3. Na recording and standard curve.

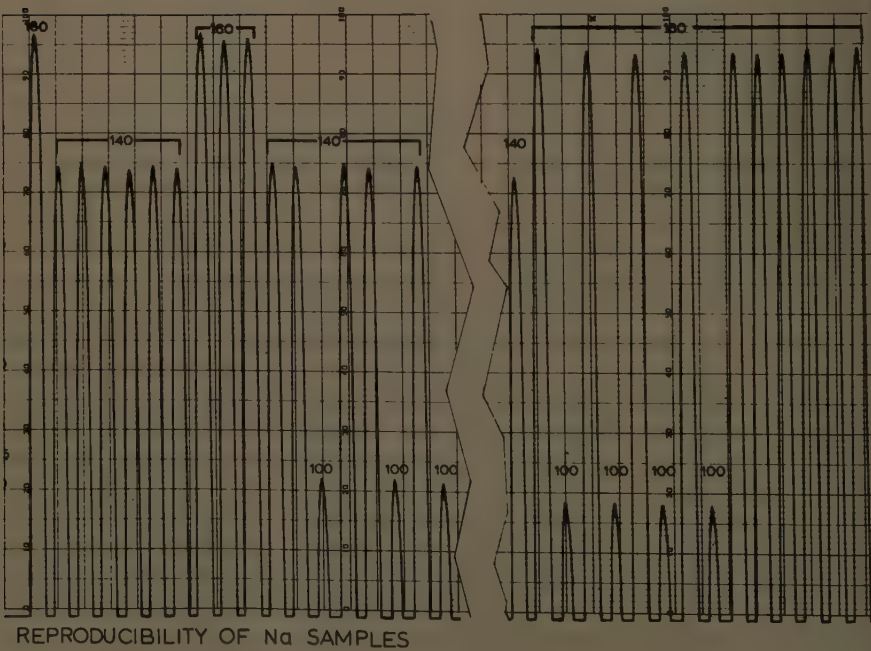
sitivity and great expansion of the recorder scale, the best individual components and the balanced ratio concept are used in the flame photometer. This method is used in the electric circuitry and in the optical portions. The balanced ratio system minimizes noise on the record from minute variations in gas pressures, dialysis rates, sample flow rate, flame artifacts, and power supply variations.

FIGURE 8 shows an over-all view of the flame photometer module. The lower base box contains an air filter that removes all particles larger than  $0.3 \mu$ . All air entering the photometer passes through the filter. So much flow resistance results that a simple air blower is required to ensure adequate flow for cooling to minimize thermal drift during warm-up and operation.



REPRODUCIBILITY OF  
Na SAMPLES

FIGURE 4a. Reproducibility of Na samples.



REPRODUCIBILITY OF Na SAMPLES

FIGURE 4b. Reproducibility of Na samples.

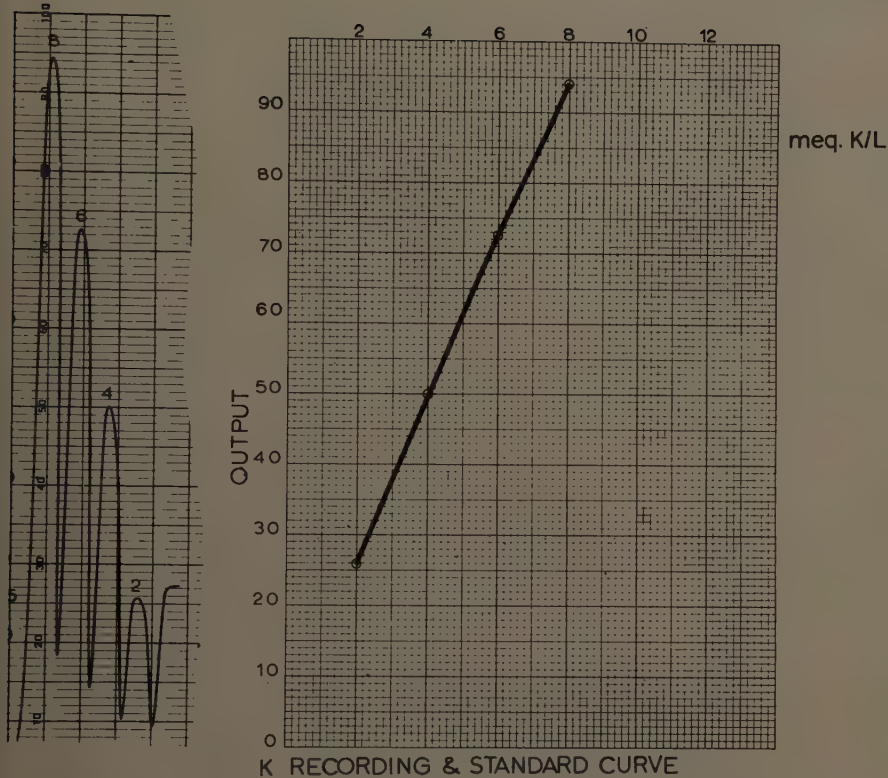


FIGURE 5. K recording and standard curve.

#### REPLICATE SERUM POTASSIUM SAMPLES

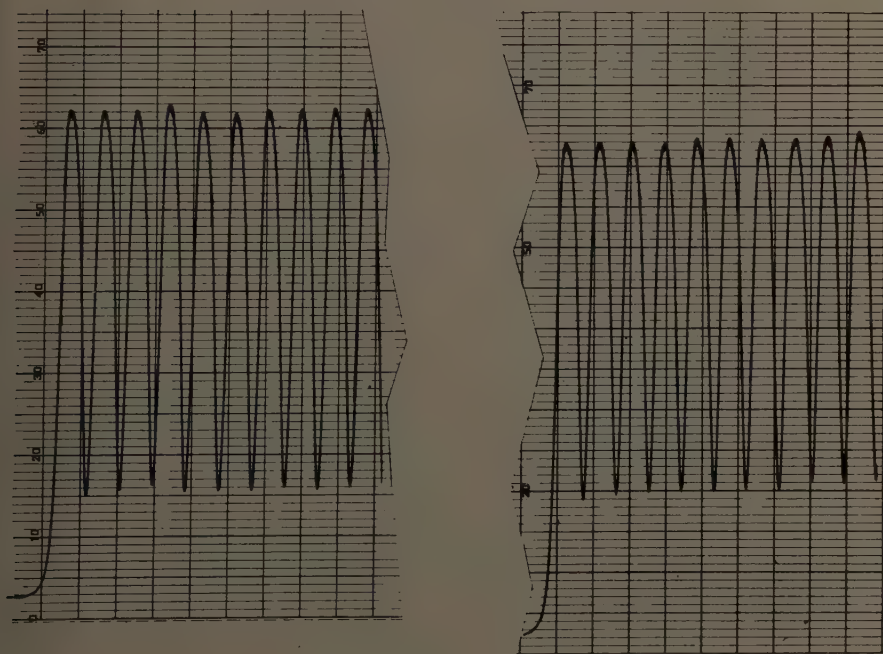


FIGURE 6. Replicate serum potassium samples.



The lower box also contains the burner and associated valving and the controls described in greater detail subsequently.

The smaller box on top of the base is the flame-integrating unit containing the photosensitive cells. This unit can be lifted at will from the base. The top chimney provides a duct for escape of hot gases while preventing entrance of external light and vagrant particles, and it maintains a comfortable auditory noise level. The controlled flow of hot gases upward and outward prevents counterflow of external air and particles and also helps keep the flame photometer cool.

Gas supplies of propane and oxygen are used because they permit the

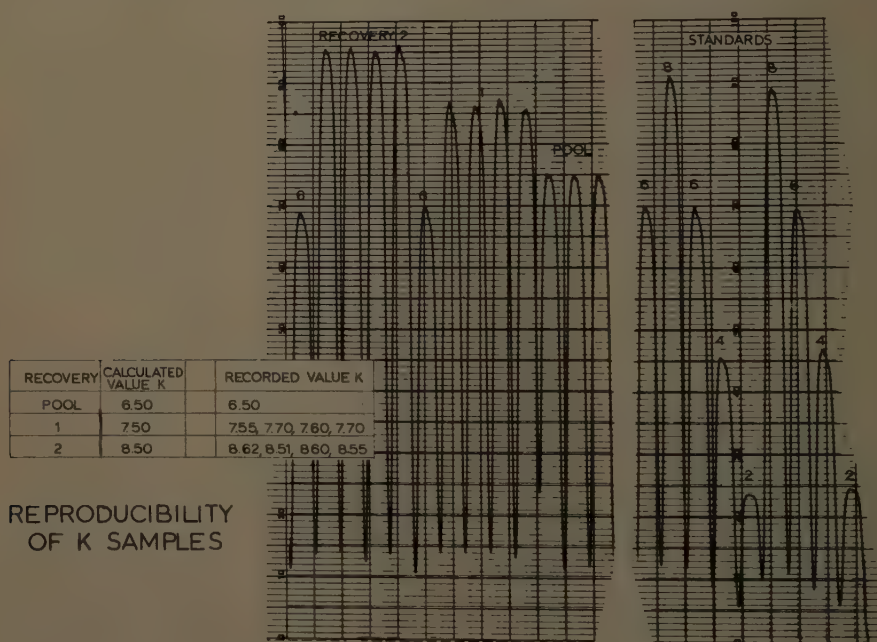


FIGURE 7. Reproducibility of K samples.

least restrictive installations and offer the greatest safety in a plant or laboratory. The flame temperature of propane-oxygen is somewhat lower than that of hydrogen-oxygen, as shown on FIGURE 9,<sup>4</sup> and this decrease has some value in analyses of Na and K with Li as an internal standard.<sup>3</sup>

The burner is shown in FIGURE 10. It operates along the general principles of the cyanogen-oxygen burner first reported by Vallee and Bartholomay.<sup>5</sup> The flame-front-propagation speed of a stoichiometric mixture of propane and oxygen is well below the gas-stream velocity required for sample aspiration. It is necessary, therefore, to use main and auxiliary flames in burners employing propane or other similar gases. The main flame gases issue through the fine annulus surrounding the central sample capillary at speeds sufficiently high to provide adequate atomization and evaporation.

The burner can also aspirate, but this function is of secondary importance. This main flame gas speed is sufficiently high to blow away the flame. The ring of holes around the central annulus provides the auxiliary flame gases at speeds below the flame-front-propagation speed; therefore the auxiliary flame is not blown away and keeps the main flame burning continuously.

The proportions of the main and auxiliary gases are premetered and pre-

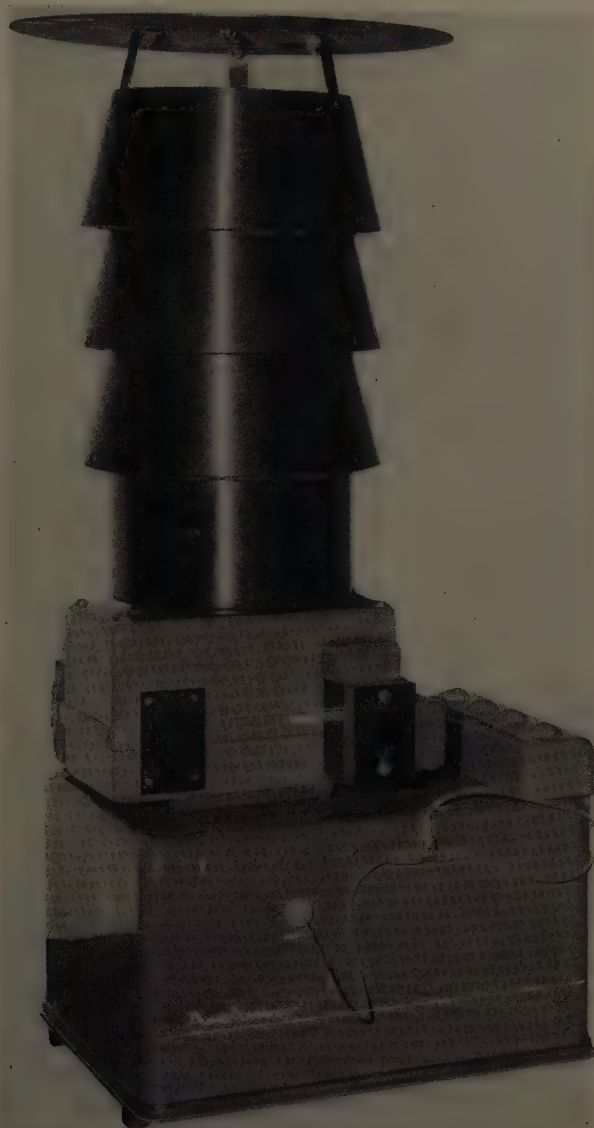


FIGURE 8. General view of flame photometer module.

TEMPERATURES OF SOME FLAMES

Fuel-oxidant mixture	Temperature, °C.	
	From Gaydon	Fom Mavrodineanu and Boiteux
Hydrogen-air . . . . .	2100	2115
Hydrogen-oxygen . . . . .	2810	2690
Acetylene-air . . . . .	2250	2050
Acetylene-oxygen . . . . .	—	3110
Methane-air . . . . .	—	1955
Methane-oxygen . . . . .	2737	2720
Ethylene-air . . . . .	—	1895
Propane-air . . . . .	—	1925
Propane-oxygen . . . . .	2776	—
Manufactured gas-air . . . . .	—	1840
Manufactured gas-oxygen . . . . .	—	2800
Cyanogen-oxygen . . . . .	4850	—

FIGURE 9. Temperatures of some flames. Reproduced by permission from Interscience Publishers, Inc.<sup>4</sup>

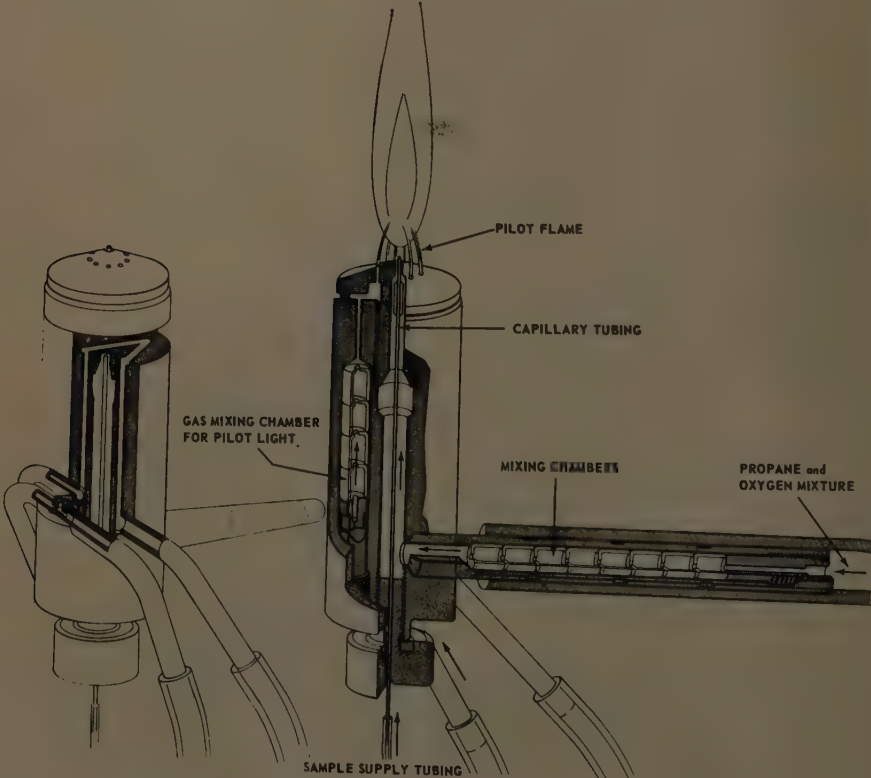


FIGURE 10. Burner.



mixed. The metering is performed by fine needle valves, the mixing by alternate compression and expansion in the mixing chamber elements. Individual needle valves are used in each flame because a more stable flame is achieved by keeping the auxiliary flame slightly rich and the main slightly lean. Once the needle valves are adjusted to provide a good flame, tank pressure regulator manipulation alone suffices to light and extinguish the flame. The needle valves do not require readjustment for many months.

Flashback of the flame into the supply lines of premixed gases is made impossible by the high jet speeds at the main flame annulus and at the small orifice just behind the auxiliary flame holes.

Attempts have been made to use only oxygen in the main flame and a very rich mixture in the auxiliary flame, but this method does not offer a flame as compact, intense, or stable as the present flame.

Because the auxiliary flame lingers on the metal surface, the burner gets hot. In the burner in FIGURE 10, water is circulated internally to keep it cool. In the future, finned burners without water circulation that will dissipate the heat into the rising column of air created by the air blower mentioned previously probably will be used.

The cap and capillary are removable for cleaning. In practice it has not been necessary to remove the cap after many months of daily operation. Neither is the capillary often removed. It has become standard procedure to run a wire through the capillary at the end of the day and forcibly to inject a short stream of water at the base of the burner. Very little residue is left on top of the burner after days of operation. A quick wipe at the end of the day is all the precaution required.

Excellent gas-pressure regulation is required. The standard tank regulators are replaced or augmented by special-purpose, specific range regulators. Sensitive pressure gauge recordings in early work showed that variations of gas pressure closely paralleled variations in sample recordings. Variations in regulated gas pressure probably lead to variations in flame temperature that affect Na, K, and Li differently. In a single-ended system the error is gross; in a ratio system the error is minimized. FIGURE 11 shows recorder output with oxygen pressure held constant and propane pressure varied for single-ended systems of Na and Li and for an Na:Li ratio system. The improvement is obvious and, of course, gas pressures do not vary as much as shown.

Aspiration of sample, as compared to positive pumping of sample, leads to errors in delivery to the flame resulting from myriad variations in such factors as apertures, pressures, densities, and viscosities. For each metal determination in a given system, a specific sample flow rate exists which produces maximum emission intensity.<sup>6,7</sup> The degree of ionization and of cooling of the flame by the solvent, generally water, are involved. Judicious compromises are required when the solvent contains a number of metals. Direct fixed-flow-rate pumping assures better control of these factors.

Work is planned for evaluation of the present burner for higher temperature flames such as the cyanogen-oxygen flame.

The burner is located in the central portion of the integrating chimney as shown in FIGURE 12. The chimney is made of glass and is surrounded

by a fine powder of magnesium oxide (MgO). It has been selected for its crystalline structure and its very high reflectivity of 98 to 99 per cent of incident light. The powder is retained in place by a housing. Aluminized chimneys have been used, but are substantially inferior for the purpose.

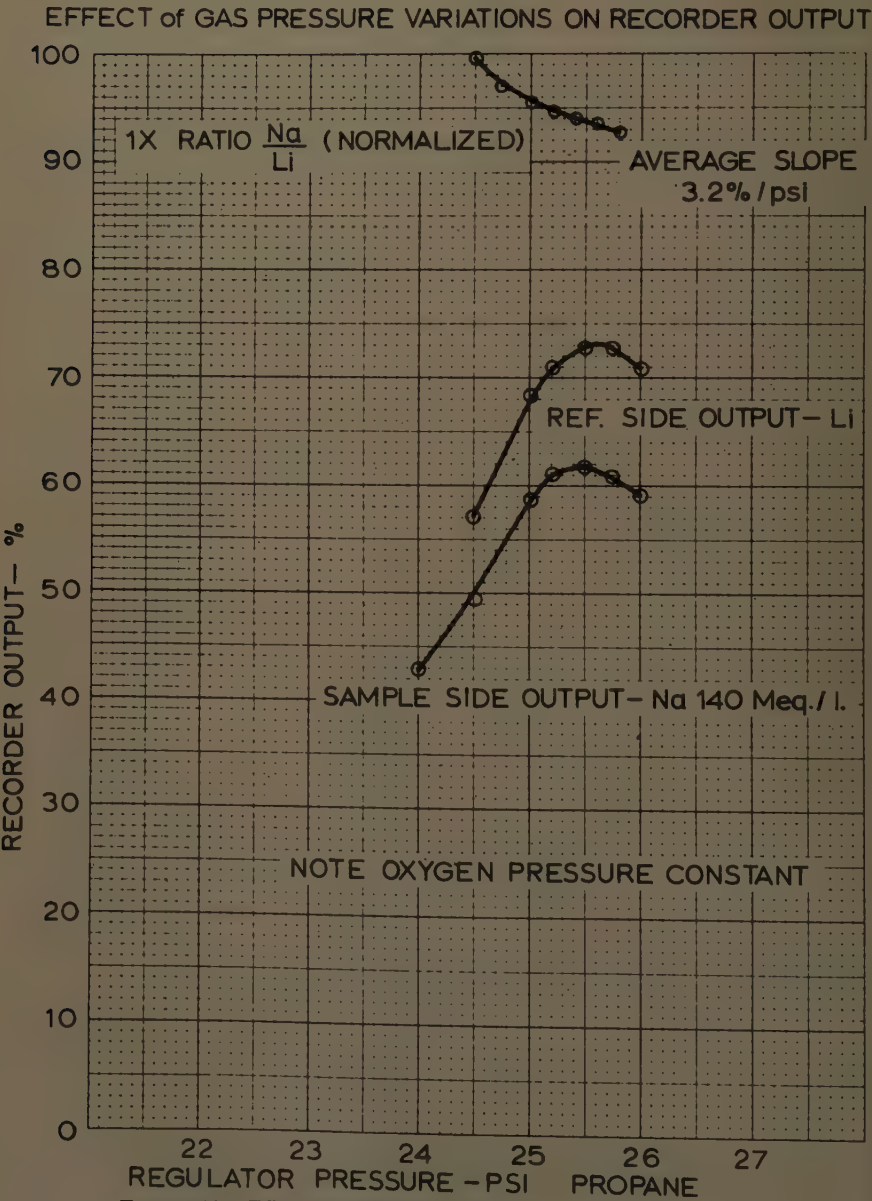


FIGURE 11. Effect of gas pressure variations on recorder output.

The flame burns within the chimney, and a major portion of the light is diffused, reflected, and integrated by the shape of the chimney and the random arrangement of crystals. The light finally enters each end of the side sight tubes where the photosensitive detectors must necessarily see precisely the same amount of light flux. These cells do not see the flame directly.

Flickering in portions of the flame is integrated and seen equally by both detectors. By use of a balanced ratio system, the minute variations in such factors as the flame, gas flows and pressures, dialysis rates, sample flow rate, and power supply are minimized. The improvement of the double-ended

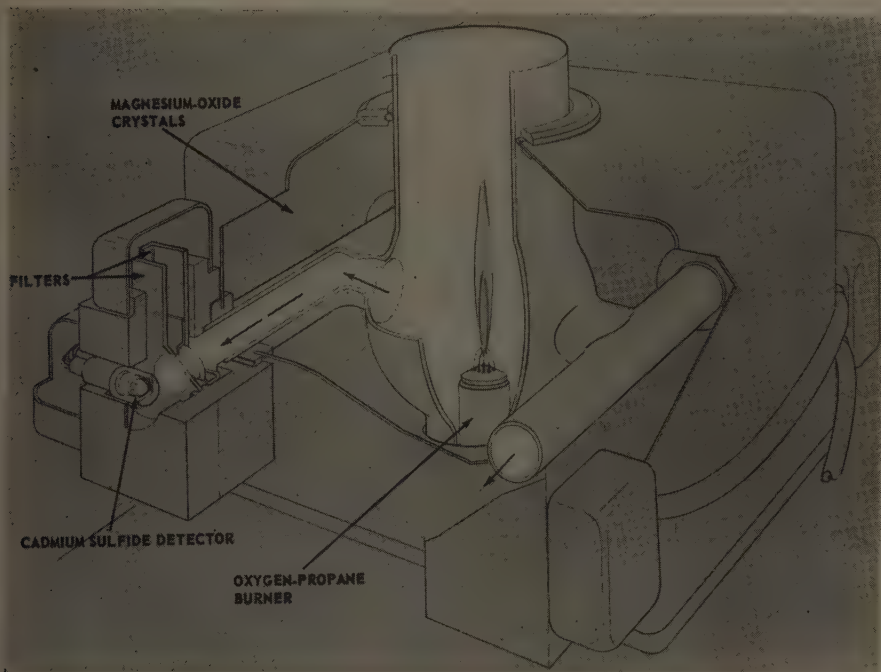


FIGURE 12. Integrating chimney.

ratio system over a single-ended system, for the factors mentioned, is of the order of 5 or 10 to 1.

Interference filters coupled to glass blocking filters, in light-tight housings, are placed at each end of the sight tube. The peak analytical wave lengths used for sodium, potassium, and lithium are  $589\text{ m}\mu$ ,  $767\text{ m}\mu$ , and  $670\text{ m}\mu$ , respectively.

The use of these sharp optical filters prevents unwanted emission lines and extraneous light from reaching the cells. Thus the electric output due to flame background and interfering substances in the flame is negligible.

The electronic system consists of a reference detector and a sample detector arranged in a ratio null balance circuit as shown in FIGURE 13.

A standard concentration of sample is introduced into the system together



with the  $\text{LiNO}_3$  for calibration purposes. The latter is used with either Na or K as a constant reference.

A Zener regulated power supply energizes the detectors. The reference side current is proportional to the lithium concentration; the sample side, to the sample concentration. The ratio of the two sides is shown on the recorder. Thus if the light intensity changes, the absolute values of the voltages developed change, but the ratio of the two remains the same and the reading on the chart stays constant.

In a ratio system good noise-cancelling ability requires that each of the detectors respond proportionately the same amount to a small increment of

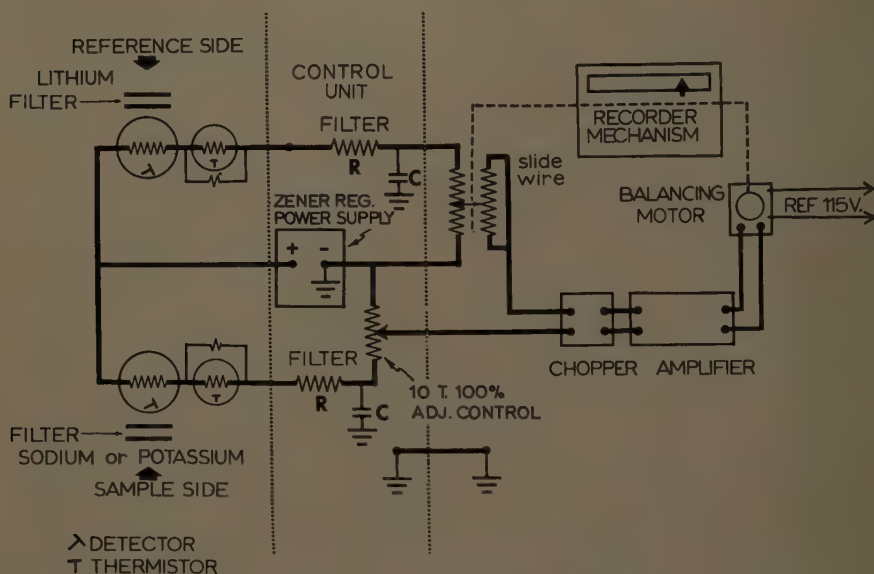


FIGURE 13. Electric block diagram.

illumination. Linearity-matching of cells and y-intercept suppression further optimize the effectiveness of the balanced ratio system.

Photoconductive cadmium sulfide cells are used as the light-sensing elements because of their small size and high sensitivity. Photocells and phototubes are not sufficiently sensitive to be used. The spectral response of the cadmium sulfide cell is sufficiently wide to permit its use for the Na, K, and Li wave lengths. When energized by a constant voltage supply, the output current-illumination plot is not a straight line. However, the deviation from linearity is slight for the K curve or the clinical range of the Na sensitivity curve (FIGURES 3 and 5). Note that the upper 25 per cent of the sodium curve is expanded to full scale.

Due to the high sensitivity of the system, the detector circuit requires temperature compensation. Thermistors are incorporated into a copper slug together with the detector to maintain the cell and the compensating element at the same temperature. Shunt resistors are adjusted so that the de-

tector-thermistor combination has a minimum coefficient over the normal temperature range. Since the temperature coefficient (about 1 per cent/°C.) of the cells is a function of illumination level and wave length, illumination is equalized by means of neutral density filters.

Numerous schemes have been used at the ends of the sight tubes in the integrating chimney. It is possible to place a cell with its companion thermistor at each end of the sight tube, one cell for sample determination, the other for reference. It is further possible with the present photoconductive cells to place both sample and reference cells with companion thermistors at the same end of the sight tube. This arrangement is preferred for compactness and temperature compensation. By placing sets of cells at each end of a sight tube two determinations can be made simultaneously from the same sample. The use of a 2-point recorder and associated circuitry is then required.

In automatic, continuous, or repetitive unattended flame photometry, the variations permissible in manual determinations because of continual manual recalibration must be minimized to the vanishing point. The program for the present equipment has revolved largely, therefore, around the development of more precise and reliable system arrangements and components than previously available. It has been equally important to cancel the effects of environmental variations. The present equipment is excellent, and efforts continue to make it better. The integrating flame photometer provides continuous and repetitive automatic determinations when used in conjunction with the AutoAnalyzer.

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## THE DETERMINATION OF CARBON DIOXIDE IN BLOOD SERUM

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The first practical apparatus for the determination of blood gases was developed by Barcroft and Haldane in 1902.<sup>1</sup> In this apparatus a sample of blood was placed in a large enclosed chamber filled with atmospheric air and treated first with alkaline ferricyanide to cause the release of oxygen and then with tartaric acid to cause the evolution of carbon dioxide. The amounts of each of these gases released into the supernatant air was measured by either volumetric or manometric means. The major fault of this apparatus was the very large volume in the chamber, which required very careful temperature control and a long equilibration period in order that the gases be measured with accuracy. The apparatus described by Van Slyke in 1917 represented a very marked improvement.<sup>2</sup> With this equipment, the blood gases were extracted rapidly by means of a vacuum after appropriate chemical treatment, and were then compressed and measured volumetrically. Van Slyke and Neill<sup>3</sup> in 1924 greatly increased the precision of this apparatus by substituting manometric for volumetric measurement. Although many microgasometric techniques have been described, the method of Van Slyke is still the standard method in most laboratories.

In recent years, however, many laboratories have found that their work load has increased considerably, while the number of technicians available to perform the additional tasks has not. Unfortunately, the method of Van Slyke is not rapid and cannot be accelerated by analysis in large lots, as can be done with most conventional colorimetric methods. It was felt to be highly desirable, therefore, to adapt the determination of carbon dioxide in plasma to a completely automatic system of analysis such as the AutoAnalyzer.<sup>4</sup> The AutoAnalyzer has been quite successful in the determination of urea, sugar, chlorides, and other elements in blood for which a direct colorimetric test is available. There is no specific colorimetric reaction for carbon dioxide, so that carbon dioxide determination cannot be adapted directly to the automatic colorimetric method. It was found, however, that carbon dioxide is released rapidly from a flowing acidified stream of plasma and that it may then be transferred continuously to and absorbed in a second stream of alkaline-buffered phenolphthalein that is suitable for its continuous colorimetric determination.

A method utilizing these principles has been described recently.<sup>5</sup> Samples are aspirated from the turntable pickup device (FIGURE 1) by means of a multiple proportioning pump at the rate of 0.32 ml./min., and are mixed continuously with  $\frac{1}{10}$  N lactic acid containing a small amount of silicone Antifoam.\* The stream is segmented at this point by the addition of bubbles of carbon dioxide-free air. The combined stream then is passed through two helical mixing coils and discharged into a small glass trap. While the

\* Dow Corning Antifoam B, Dow Corning Corporation, Midland, Mich.



stream passes through the mixing coils, carbon dioxide is liberated from the acid liquid mixture and enters the air bubbles. Since carbon dioxide has a small but real solubility in water, one might expect that some time would be required for equilibration of the carbon dioxide between the gas and liquid phases. In practice, however, any equilibration period required must be very short, since elimination of one or even both of the mixing coils makes very little difference in the final results. After discharging into the trap, all of the liquid and one half of the gas mixture are discharged to waste. The remaining one half of the gas mixture is aspirated once more by the pump and used to segment a stream of color reagent containing an alkaline carbonate-bicarbonate buffer and phenolphthalein. This colored stream then is passed through two mixing coils where carbon dioxide is absorbed in the al-

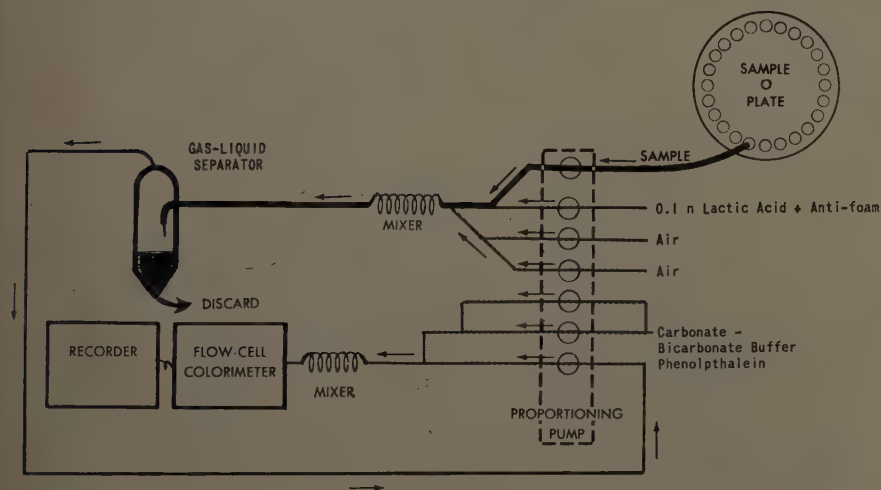


FIGURE 1. Schematic flow diagram of the method of automatic carbon dioxide determination.

kaline solution, the *pH* of which is thus lowered and the indicator partially decolorized. The stream is directed finally to a recording flowcell colorimeter having a 6-mm. cell depth and a 550-m $\mu$  light filter.

A typical recording taken from the apparatus shows a series of excursions from a base line of about 25 per cent transmission (FIGURE 2). Each excursion represents an individual sample. Since the presence of carbon dioxide decolors the reagent, the excursions are in the direction of 100 per cent transmission, and inverse colorimetry is employed. The entire apparatus is calibrated by analyzing standard solutions of sodium carbonate. The response to standard solutions containing 10, 20, 30, and 40 mEq. CO<sub>2</sub>/l. are shown at the right of FIGURE 2. The peak transmission values may be plotted on semilogarithmic paper and the value of the unknown samples obtained in the usual way.

The sensitivity of the method can be adjusted easily by varying the buffering capacity of the color reagent (FIGURE 3). This is done most conven-

iently by changing the amount of a stock buffer solution (sodium carbonate and sodium bicarbonate in a molar ratio of 1:2) incorporated in the solution. For the determination of carbon dioxide in serum, usually 4.5 ml. of buffer is used per liter of reagent, giving a spread of almost 50 transmission lines for 40 mEq. bicarbonate. If no buffer is used and the color reagent is simply an alkaline phenolphthalein solution, a very sensitive calibration curve is ob-

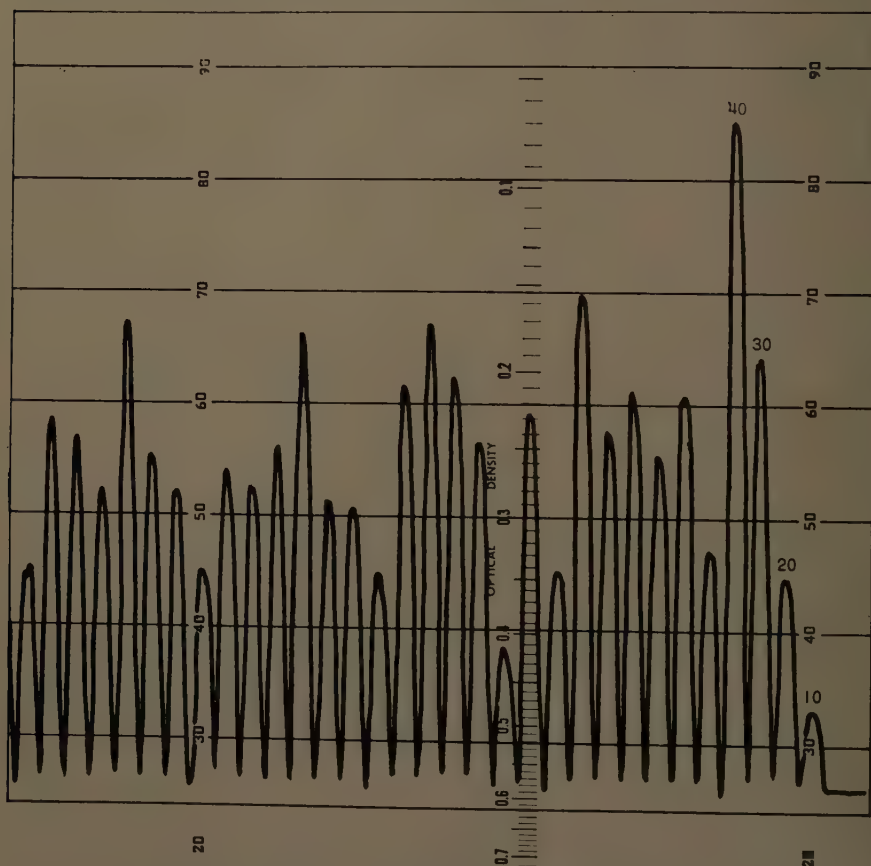


FIGURE 2. Typical recording of carbon dioxide analyses made at the rate of 40 per hour.

tained in which 10 mEq. bicarbonate causes a full-scale excursion. None of the calibration curves are straight lines, but they are easily reproducible and cause no difficulty in calculation of the results.

Other buffer-indicator solutions have been used in place of the carbonate-bicarbonate-phenolphthalein mixture. Considerable work was done with mixtures of sodium bicarbonate and phenol red. The pH of the sodium bicarbonate solution was determined by the partial pressure of the carbon dioxide in the air with which it was in direct contact. This presented a consid-

erable difficulty when the gas-liquid mixture was discharged into the colorimeter cell where the supernatant gas would accumulate and change the pH of solutions flowing in subsequently. The washout of succeeding samples was very poor. Buffers having a higher pH are to be preferred because of their lower carbon dioxide tension. Mixtures of carbonate-bicarbonate buffer and the indicator thymolphthalein were also used. This proved to

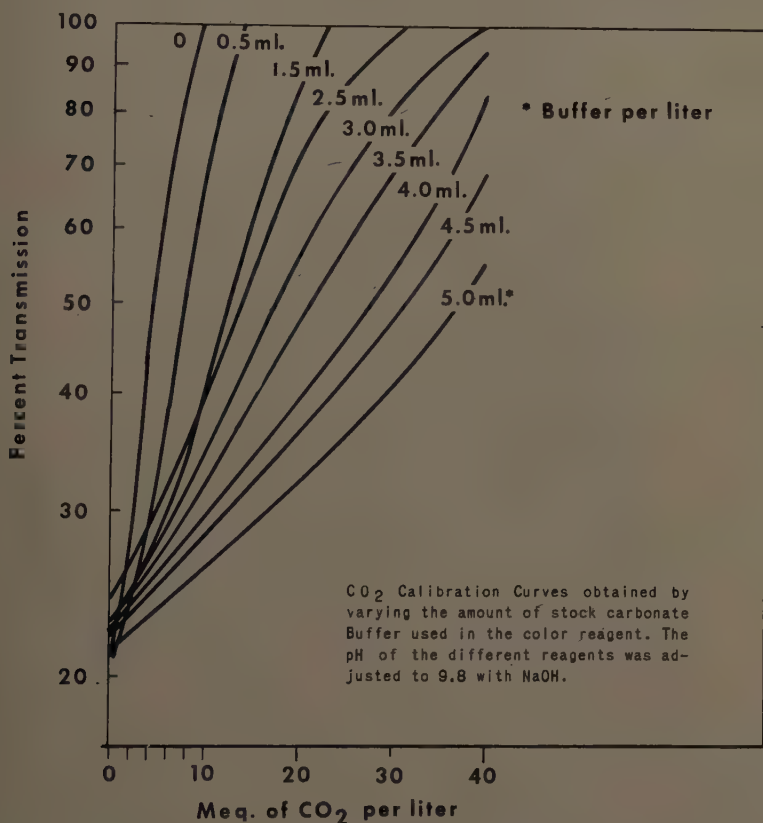


FIGURE 3. Calibration curves for the automatic method of carbon dioxide analysis.

be quite satisfactory, except that the insolubility of the acid form of the indicator in water necessitated the addition of large amounts of alcohol. Phenolphthalein does not suffer from this disadvantage.

It was necessary to verify the automatic method by direct comparison of the results with those obtained with Van Slyke's conventional manometric apparatus. In one comparison of the two methods in which 50 samples in all were analyzed by both methods, an average difference of 0.76 mEq./l. was found.

The method may be used for the determination of both plasma and serum

carbon dioxide content and also combining power. To determine the carbon dioxide content, it is necessary that the blood samples be obtained by any of the acceptable anaerobic techniques. The samples then are transferred to the small plastic cups of the pickup device and immediately layered with mineral oil. The mineral oil is lightened by dilution with 20 per cent hexane. With this protection, the samples may stand for as long as two hours prior to analysis without significant loss of carbon dioxide.

The recognized methods of equilibration of serum samples with alveolar air prior to the determination of carbon dioxide-combining power are tedious and time-consuming and require that each sample receive individual attention. For this reason, an apparatus was built which allows all samples to

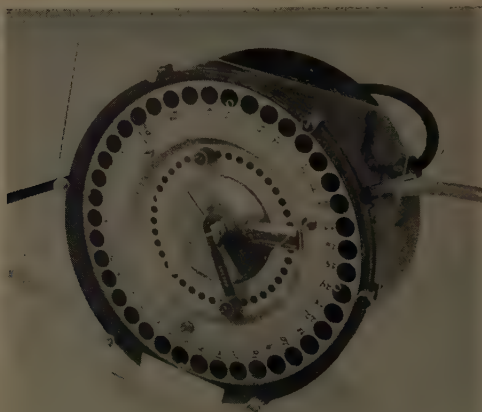


FIGURE 4. Apparatus for equilibration of plasma samples with artificial alveolar air.

be equilibrated rapidly and simultaneously (FIGURE 4). One half- to 1-milliliter portions of samples are poured into small plastic cups and inserted into the plate from the sample pickup device. The plate is then placed on a motor-driven spindle projecting from the bottom of a circular box. The box is covered with a plastic plate and placed on its side in a nearly vertical position, and the motor is started. As the plate rotates within the box, the individual serum samples are caused to flow around the inner surfaces of the cups, exposing large and changing surfaces for equilibration. During the period of rotation, an artificial alveolar air mixture is caused to flow through the box at the rate of 5 l./min. for 3 min. and 1.5 l./min. for 15 min. At the end of this time, all the serum samples are completely equilibrated with the air-carbon dioxide mixture. The box is returned to a horizontal position and two drops of oil are placed over the surface of each sample through



a small hole provided for the purpose. The plastic plate is removed, the plate is transferred to the pickup device, and the analyses are begun.

It is worthwhile to determine chloride on any sample for which a carbon dioxide analysis is requested, and vice versa. Only one sample plate need be loaded for both of these determinations. The samples are analyzed for their chloride content, and then the plate is equilibrated with alveolar air, as described above, and reanalyzed for its carbon dioxide content. In addition to saving time, this expedient has the advantage of providing an additional and related determination.

In addition to providing a rapid and automatic method for the determination of carbon dioxide in individual serum samples, the present method may also be used for continuous analysis. Thus it is possible to monitor the carbon dioxide content of the blood of a patient undergoing treatment, or of an animal during an experimental procedure. None of the methods

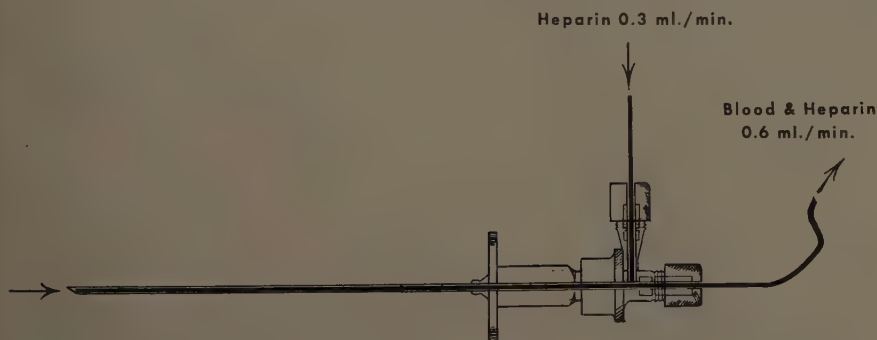


FIGURE 5. Hypodermic needle adapted for the aspiration and heparinization of a sample continuously withdrawn from a blood vessel.

previously described for the determination of carbon dioxide permit such applications.

One problem peculiar to continuous analysis is the necessity for withdrawing blood from a vein or an artery over a long period of time without the occurrence of clotting. This problem can be solved by a procedure devised by Duncan Holaday in which a double-lumen needle or catheter is used (FIGURE 5). A very dilute heparin solution (20 mg./100 ml. saline) is pumped at the rate of 0.3 ml./min. through the outer lumen of the needle. Blood is aspirated, together with all of the heparin, through the inner lumen of the needle at the rate of 0.6 ml./min. With this system, no heparin enters the blood stream of the patient, and the sample may be withdrawn continuously at the rate of 0.3 ml./min. for long periods of time without the occurrence of clotting.

Several experiments have been conducted to determine the practicality and value of the continuous method of analysis. In one experiment with a patient with chronic pulmonary emphysema (FIGURE 6), the initial carbon dioxide content was between 25 and 27 mEq./l. and was subject to small but

unexpected oscillations. Oxygen therapy was instituted and resulted in a gradual fall of the carbon dioxide content to approximately 22 mEq./l., at which value it remained steady for a considerable length of time.

Another experiment was conducted on a patient with "salt-losing" nephritis (FIGURE 7). The initial carbon dioxide content was reasonably steady at approximately 23 mEq./l. After a short base-line period, an intravenous

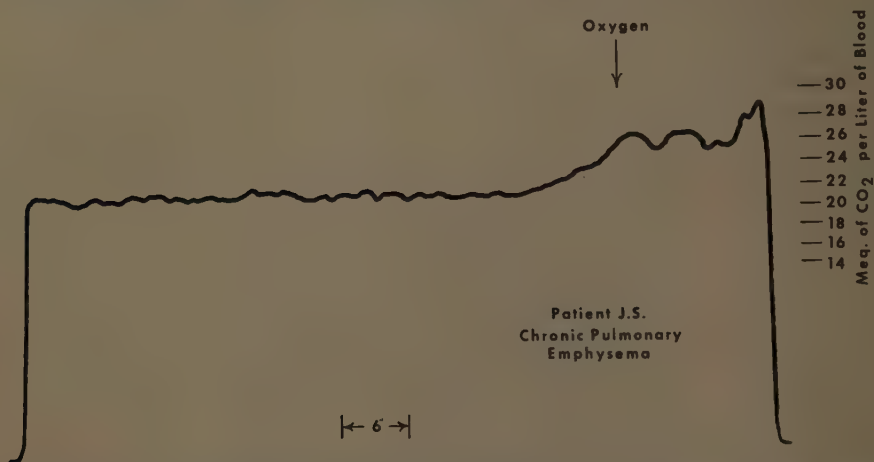


FIGURE 6. Continuous recording of the concentration of venous whole blood carbon dioxide in a patient with chronic pulmonary emphysema.

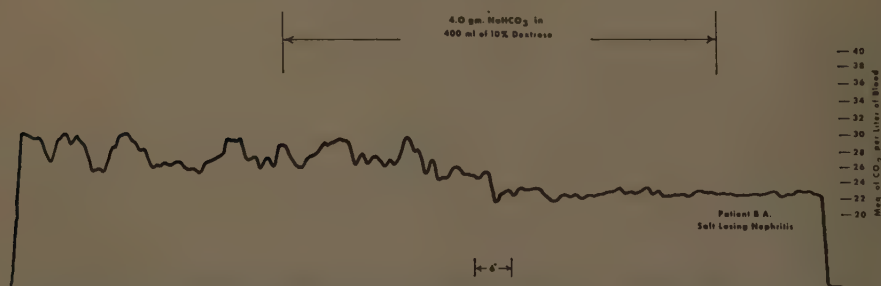


FIGURE 7. Continuous recording of the concentration of venous whole blood carbon dioxide in a patient with "salt-losing" nephritis.

infusion of 4 gm. of sodium bicarbonate in 400 ml. of 10 per cent dextrose was started. During the first portion of this infusion, the carbon dioxide content remained unchanged. Later, it increased by about 4 mEq./l. and produced a series of oscillations that are as yet unexplained. The oscillations were roughly cyclic in character, and of 3 to 4 mEq./l. in amplitude.

It should be possible to utilize the principles embodied in this method in determinations other than the clinical determination of carbon dioxide in serum and plasma. In theory, at least, any reaction that produces carbon dioxide could be analyzed by this method or by an adaptation of it. For

example, the production of carbon dioxide from L-amino acids by specific decarboxylases has actually been accomplished by Schaiberger and Ferrari, as shown elsewhere in this monograph. Since there are many such enzymatic or chemical reactions that produce carbon dioxide, the method could be used for the determination of many substances.

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# CONTINUOUS *IN VIVO* DETERMINATION OF BLOOD GLUCOSE IN HUMAN SUBJECTS

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An instrumental system for continuous automatic colorimetric analysis, the AutoAnalyzer,\* has been described previously.<sup>1,2</sup> The unit consists of a proportioning pump for metering reagents and sample. Continuous dialysis of the sample after addition of the reagents is used to achieve separation from interfering materials. The reagent mixture is heated so that the reaction may take place, and then it passes into the continuous flow cuvette of the colorimeter, where optical densities are measured and a ratio recording is obtained.

This system was applied by Ferrari *et al.*† to continuous chemical monitoring of blood glucose concentrations, and the effect of pharmacological agents on these levels was measured in rabbits. An intravenous needle was inserted into the jugular vein and polyethylene tubing was introduced inside the needle. The tubing was tied in place and the needle removed. In the same way, a second cannula was then inserted in the vein. The first catheter was used for the continuous removal of blood and the second one, for the continuous infusion of a sedative-anticoagulant medication. The first catheter was connected through a variable-speed proportioning pump to the sampling side of the manifold. A second channel of the pump was used for the continuous infusion of the sedative-anticoagulant mixture at the same rate.

This accomplishment suggested a technique for use in human subjects. The purpose of this study was to demonstrate the feasibility of automatic, continuous determinations of blood glucose in patients, and to show the difference between the type of curve resulting and the intermittent measurements in standard use for many years.

## *Materials and Methods*

For this study, it was important to obtain a continuously anticoagulated sample of blood from the subjects. This was accomplished by using a modified double-lumen needle. This needle, originally proposed by Duncan A. Holaday,‡ was made of metal, and modification was required to eliminate its positional sensitivity.

A double cannula was constructed from polyethylene tubing, as illustrated in FIGURE 1. On the side arm of the glass H was attached a length of 0.034

\* Technicon Instruments Corp., Chauncey, N. Y.

† See article elsewhere in this monograph.

‡ Associated with the Department of Anesthesiology, University of Chicago, Chicago, Ill., and the Becton, Dickenson Co., Rutherford, N. J.



$\times 0.060$  (i.d.  $\times$  o.d.) polyethylene tubing. Through this tubing, sterile heparin solution (20 U./ml.) was metered at a rate of 0.32 ml./min. This solution was then fed through  $0.030 \times 0.048$  polyethylene tubing into which had been inserted an  $0.011 \times 0.024$  tube. The inner tube was placed approximately one eighth of an inch from the end of the outside tube. The heparin solution flowed around this inner cannula. The heparin solution flowed around this inner cannula. The inner cannula in turn was connected by  $0.034 \times 0.060$  polyethylene tubing to a manifold pump

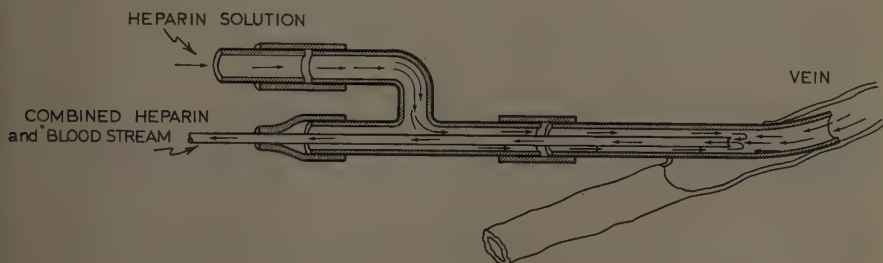


FIGURE 1. Polyethylene double cannula for continuous sampling.



FIGURE 2. Cannula inserted in lower hand.

tube that removed a total volume of 0.8 ml./min. The net difference between the flow rates of heparin being pumped in (0.32 ml./min.) and solution being removed (0.8 ml./min.) was 0.48 ml./min., or 28.8 ml./hour. The latter was the volume of blood automatically withdrawn from the patient. The heparin solution never entered the patient's systemic circulation, as it served only to prevent coagulation as blood was withdrawn from the subject. The plastic



FIGURE 3. Instrumental system under experimental conditions.

cannula not only allowed the subject greater freedom of movement of his arm but was much easier to insert in the vein. The cannula was inserted on the lower hand or forearm as follows: a thin-walled No. 16 intravenous needle with a stylet was introduced into the subject's vein, the stylet was removed, and the cannula, previously sterilized with aqueous Zephiran solution (1:1000), was threaded through the needle and into the vein. The needle was then withdrawn, leaving the cannula inserted in the blood vessel as illustrated in FIGURE 2.

The method employed for the determination of glucose was a modification of the one proposed by W. S. Hoffman.<sup>3</sup> Glucose was measured on whole blood by using a direct-reading procedure involving the potassium ferricyanide-potassium ferrocyanide oxidation-reduction system. The anticoagulated specimen was diluted with 1.6 ml. of 0.5 per cent potassium cyanide in 0.9 per cent sodium chloride. At the same time, the solution was segmented with air. This mixture entered the dialyzer and was dialyzed against 3.4 ml./min. of an 0.075 per cent potassium ferricyanide solution made up in 2 per cent sodium carbonate and 0.9 per cent sodium chloride. The dialyzed sample in the ferricyanide-cyanide solution passed through a heating bath which facilitated the reaction. The amount of yellow potassium ferricya-

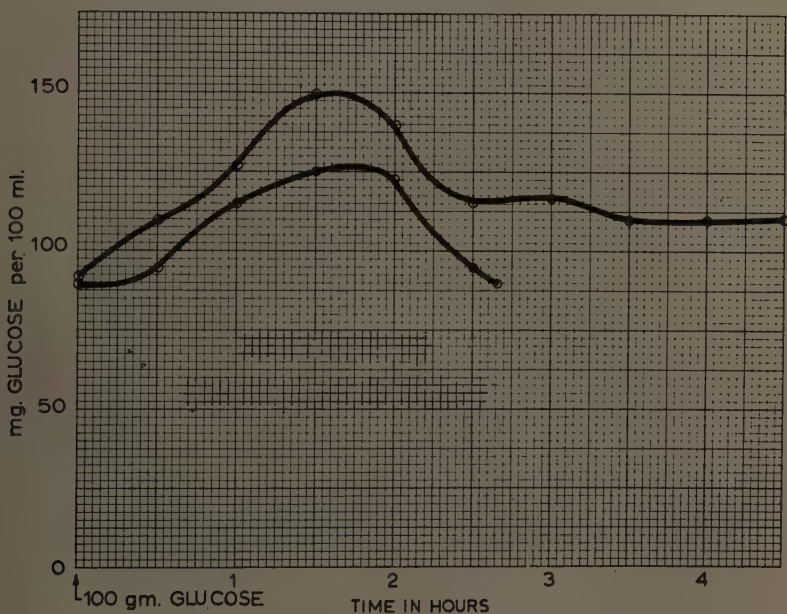


FIGURE 4. Oral glucose tolerance; no diabetic histories.

nide solution subsequently reduced to the colorless ferrocyanide was proportional to the glucose concentration. The cyanide acted as a sensitizing agent in this system. The color intensity was measured at 420  $m\mu$  in the continuous-flow cuvette with a 6-mm. light path.

FIGURE 3 shows the entire equipment for the experiment including the AutoAnalyzer on a mobile cart; the technician indicates a continuous tracing that is being recorded. Normal patients, that is, individuals whose carbohydrate metabolism was not disturbed, as determined by previous studies, were selected as controls. In a standard glucose tolerance test 100 gm. of glucose was given orally at zero time after an overnight fast and continuous glucose curves were recorded. Mild, stable, diabetic patients then were selected and, in like fashion, continuous glucose determinations were obtained following oral administration of the standard amount of 100 gm. of

glucose. An insulin tolerance test was performed on a patient with Cushing's syndrome, by giving 10 units of regular insulin at zero time and measuring the effect of the insulin on the blood glucose continuously for 3 hours. Similarly, oral hypoglycemic agents were given to selected normal and diabetic individuals, and their effects on the continuous blood glucose curves were recorded.

### Results

FIGURE 4 shows the glucose tolerance curves for 2 individuals without history of diabetes after being given 100 gm. glucose. These continuous curves do not differ appreciably from the intermittent manual measurements.

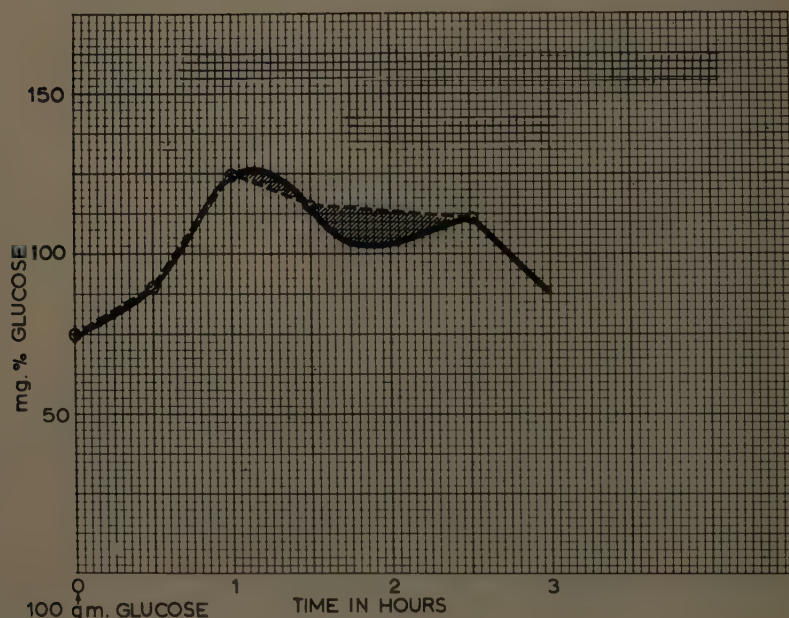


FIGURE 5. Normal oral glucose tolerance for a 26-year-old, obese man with no diabetes. —, Continuous record; --, manual values.

FIGURE 5 shows the glucose tolerance of a 26-year-old, obese man with no history of diabetes who was given 100 gm. of glucose. It will be noted that by the continuous test his glucose tolerance was higher at the end of 1 hour, and lower at the end of 2 hours, than indicated by the standard test in which, since only a number of points of the curve are determined, peaks or minima may not be recorded.

W. R. was a 64-year-old asthenic patient with stable diabetes of 10 years' duration. He was previously controlled on 15 U. NPH insulin daily; on the day of the test the insulin was withheld, and 100 gm. of glucose was given at zero time. In the 3-hour record of his glucose tolerance (FIGURE 6) very little difference between the continuous recordings and the corresponding manual



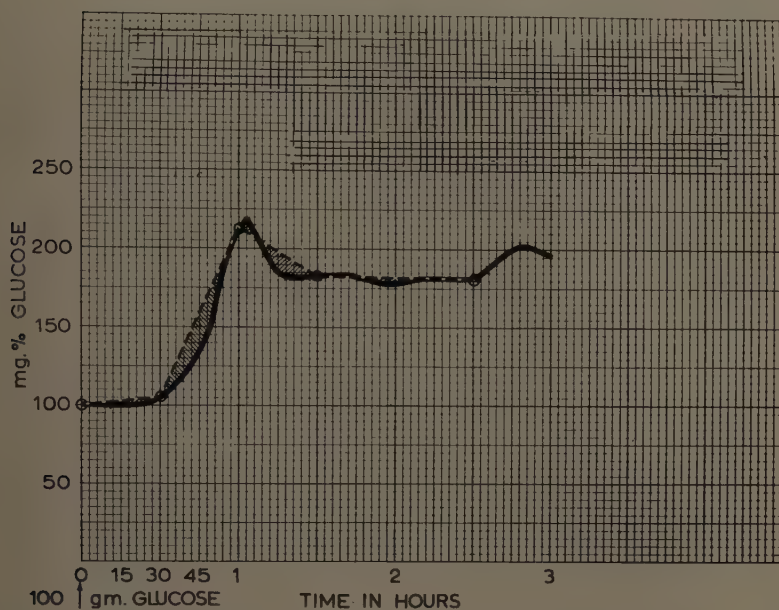


FIGURE 6. Glucose tolerance of a 64-year-old asthenic patient with diabetes of 10 years' duration. —, Continuous record; --, manual values.

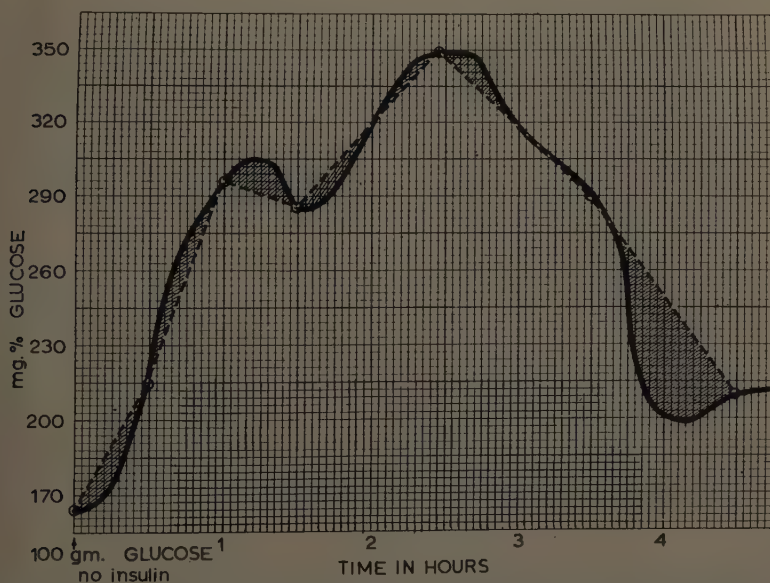


FIGURE 7. Glucose tolerance of a 38-year-old, labile asthenic woman with diabetes of 13 years' duration, normally on 30 to 45 U. NPH insulin. —, Continuous record; --, manual values.

values can be observed except for a slower rise during the second half hour and a more rapid drop during the third half hour in the continuous record.

A. L., 1 38-year-old labile, asthenic woman with diabetes of 13 years' duration, had been controlled on 30 to 45 U. NPH insulin daily. On the morning of the test, insulin was withheld and 100 gm. of glucose given orally. The resulting manual and continuous records (FIGURE 7) differ noticeably.

D. O., a 39-year-old woman with Cushing's syndrome, was aglycosuric and had no symptoms referable to diabetes, but had a diabetic-type curve in a standard glucose tolerance test. She was given 10 U. of regular insulin and its effect on her glucose curves was recorded (FIGURE 8). There was a difference

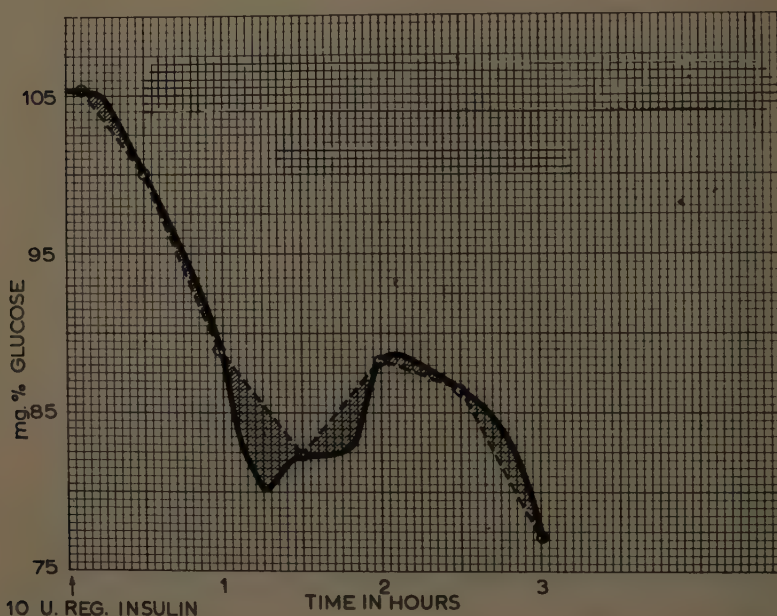


FIGURE 8. Insulin tolerance of a 39-year-old, aglycosuric woman with Cushing's syndrome but a diabetic reaction to a standard glucose tolerance test. —, continuous record; --, manual values.

between the continuous chart recording and the corresponding manual values, during the first and second hours.

D. L., a 25-year-old, obese male recently had become diabetic. He was given an injection of 10 U. of regular insulin and his glucose tolerance was followed for  $6\frac{1}{2}$  hours (FIGURE 9). This is one of our earlier runs and we were trying to record the effect of insulin on the blood glucose and establish techniques. Note the time lag between the administration of carbohydrate and the rise in the blood sugar curve; this is due to the combined effects of the time required for gastrointestinal absorption and the 8-min. lag in the recording system. The same patient was given 200 mg. of metahexamide, an oral hypoglycemic agent, and his blood glucose curve was followed for  $6\frac{1}{2}$  hours (FIGURE 10). His response was good, being similar to that obtained with insulin, although delayed.

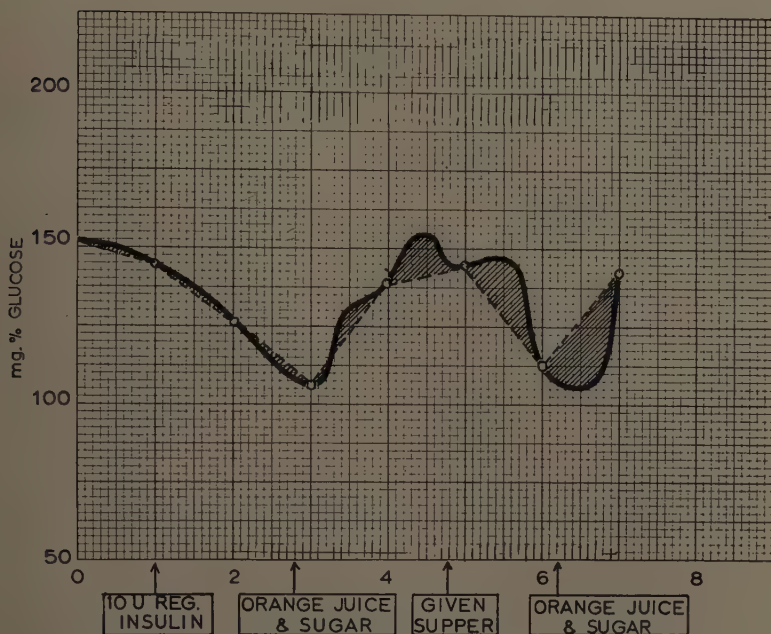


FIGURE 9. Effect of regular insulin on a 26-year-old, obese man, a new diabetic.

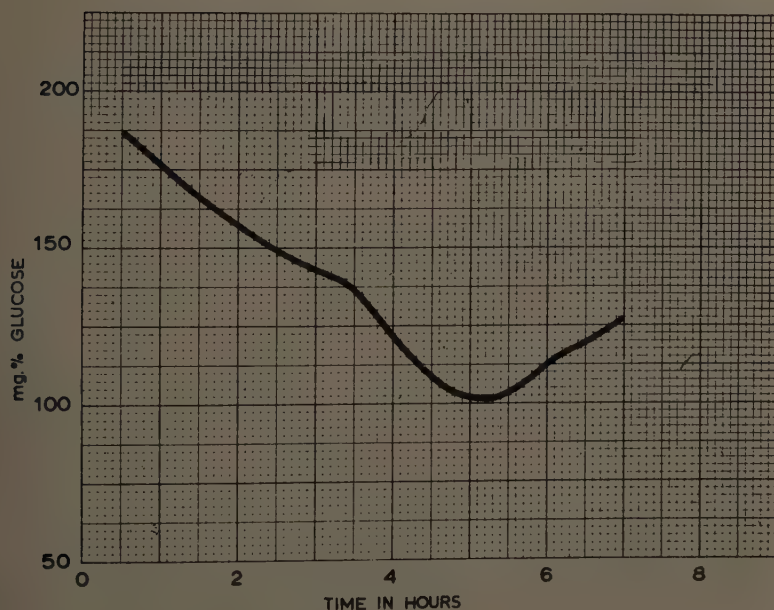


FIGURE 10. Effect of metahexamid on 26-year-old, obese man, a new diabetic.

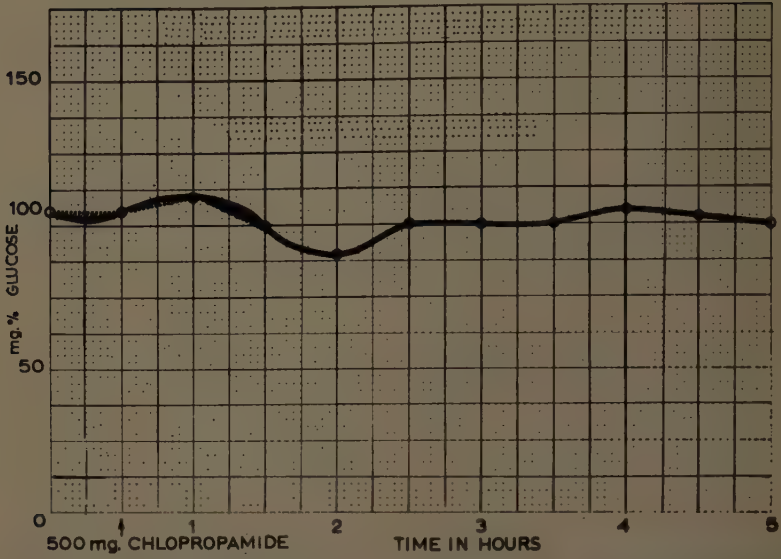


FIGURE 11. Effect of chlorpropamide on a 33-year-old man who had suffered a post-traumatic cerebral vascular accident. —, continuous record; --, manual values.

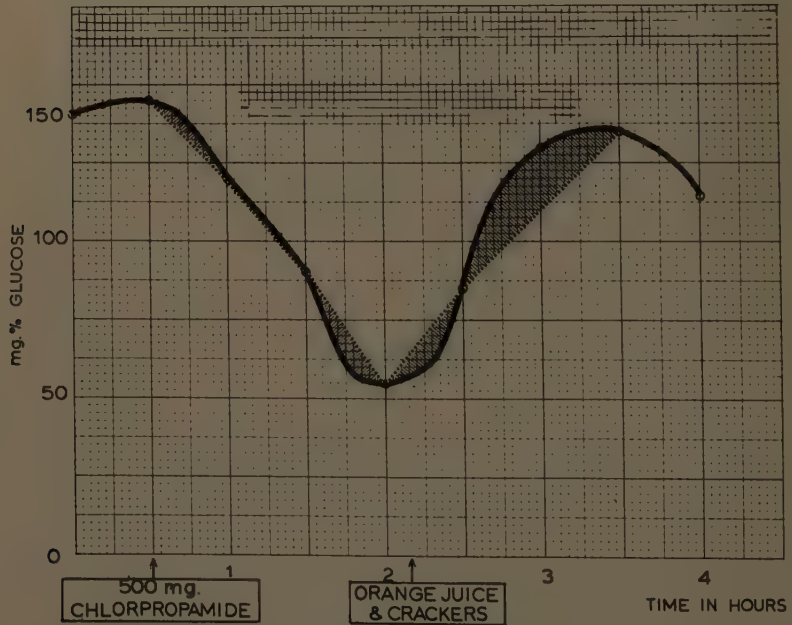


FIGURE 12. Effect of chlorpropamide on a 73-year-old diabetic man of normal weight. —, continuous record; --, manual values.



Patient L. P., a 32-year-old male who had suffered a posttraumatic cerebral vascular accident, was given 500 mg. chlorpropamide, another oral hypoglycemic agent, and his blood was glucose-measured over a 5-hour period. As may be seen in FIGURE 11, the blood glucose response of this patient to this drug was slight.

A 73-year-old, mildly diabetic male of normal weight was given 500 mg. of chlorpropamide. As shown in FIGURE 12, his blood sugar dropped to 55 mg. per cent within 2 hours and he had clinical signs and symptoms of hypoglycemia. An hour after he was given orange juice and crackers his blood glucose returned to its previous high level.

Although some of the curves show no dramatic differences between intermittent and continuous blood glucose measurements, in some instances the latter type did yield more time data than would have been obtained by the standard methods.

### *Discussion*

The development of this new technique has opened up further horizons for exploration. Although the initial investigation reported herein was limited to the continuous, intravenous analysis of blood glucose, it is possible to study other constituents in the blood and body fluids. We plan to measure simultaneously the glucose, inorganic phosphate, and drug concentrations in the blood following the administration of an oral hypoglycemic agent. To accomplish this, one cannula may be inserted in a vein, another in an artery, and a third in the bladder. Continuous, simultaneous measurements of arterial and venous blood and urinary glucose can give more accurate time data on the peripheral utilization of glucose and urinary thresholds than can standard methods. It is also planned to measure continuously the carbon dioxide content of the blood during anesthesia and surgery and to relate it to the blood glucose. The inherent flexibility of this method of analysis enables one to do studies in many parameters, subject only to the methods of chemical analysis and technology developed.

### *Summary and Conclusions*

An instrumental approach to continuous chemical monitoring of blood glucose concentration in human subjects was demonstrated.

This approach involved the use of an automatic colorimetric system of analysis in the chemical determinations. The use of a continuous flow of heparin through an intravascular double-lumen, metal, or plastic cannula eliminated clot formation and prevented passage of the anticoagulant into the systemic circulation.

The effects of orally administered glucose and antidiabetic compounds on continuous blood glucose curves were studied in normal and diabetic subjects.

This new technique gave more accurate time data than did the conventional method of intermittent measurements, and may form the basis for future work in the field of continuous chemical pharmacodynamics *in vivo*.

The inherent flexibility of this method of study will permit its use wherever new methods of chemical analysis and technology are developed.

*Acknowledgments*

We are indebted to Wilda Zahm and Constance Giumarro for their invaluable assistance.

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# A RANDOM SELECTION SYSTEM FOR AUTOMATIC DYNAMIC BIOCHEMICAL ANALYSIS\*

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## IMPACT OF MEDICAL AUTOMATION

The introduction of automatic methods into the techniques of the biomedical sciences is now more urgent than ever before. At present, a detrimental delay often occurs between the collection of a clinical specimen and the availability of the analytical results. In many cases such a delay nullifies the usefulness of the test for diagnostic purposes, and the results acquire a retrospective, educational, or only a confirmatory value. Granted that in some circumstances electronic computation and improved communication may reduce this lag, the chief reason for it is found in the relative slowness of manual operations in the analytical laboratory. A very extensive survey of the limitations of electronic computation techniques in the biomedical sciences, along with an extensive bibliography, was recently published by Ludley.<sup>1</sup> A forthcoming monograph of the National Academy of Sciences on the application of such techniques should be a welcome guide in a confused field where the main deterrent is still the insufficiency of cross-disciplinary training of the researchers.

Industrial mechanization and instrumental control methods have progressed at an accelerated pace ever since the turn of the century, when Frank Woolard in England conceived the idea of the automatic manufacturing plant. It is remarkable that, despite the extensive experimentation that followed, laboratory automatic instrumentation itself remained relatively stagnant. Under the impetus of industrial reorganization during the tumultuous years following World War II, automation scored fabulous achievements in the field of office and method planning and data processing, for which the first giant computers were developed. Such developments occurred so fast that the witness is often seized with a complex feeling, a mixture of awe for the achievement, bewilderment about the result, and fear for the future. Today, however, while management and industrial production are headed resolutely in the direction of complete automation, paradoxically, the source of basic laboratory information in the biomedical sciences lags behind at the stage of fragmentary automatic instrumentation and, more often than not, of manual operation.

One of the stumbling blocks in the path of progress in this field has been the slow acceptance of the necessary distinction between the requirements of automatic instrumentation and those of true automation. This distinction and its practical implications were developed at length in a previous publication.<sup>2</sup> It is sufficient for the purpose of this communication to recall the definition of automation I proposed at the Fourth International Automation Congress<sup>3</sup> in 1958: "Laboratory automation is defined, ideally, as the science concerned

\* The experimental work involved in the design of the amplifier and other electronic devices mentioned in this paper was supported by the Physics and Physiology Branches, Office of Naval Research, United States Navy, under a series of contracts granted to Paterson General Hospital, Paterson, N. J., and the Department of Anesthesiology, Columbia University College of Physicians and Surgeons, New York, N. Y.

with the techniques, methods, and principles involved at each phase of quantitative data acquisition, reduction, conversion, transmission, storage, retrieving, handling and computation by automatic instrumentation." This definition fulfills the requirements of an autonomous discipline, with its newly discovered theoretical principles, its reliance on cybernetics, its abstract concepts, its temporary but adequate philosophy, its characteristic experimental approach, and its own internal process of information feedback upon which depends its further progress. Implicitly, this definition requires that the equipment used incorporate provision for adequate coordination of the functions listed. This necessitates the creation of suitable systems somewhat more sophisticated than the usual servo-loop without which automatic instrumentation could not exist. This thesis was substantiated at length in a previous publication.<sup>4</sup>

The introduction of automation into the biomedical laboratory presents particular difficulties inherent in (1) the unique manner in which the information obtained is used and (2) the nature of the materials investigated.

The manner in which biomedical information is used results in unique difficulties. The purpose of obtaining such data is the reconstruction of a fragmentary biological picture and its insertion in a frame of biocentological concepts<sup>5</sup> by means of statistical associations. This does not necessarily imply causal relationships between the fragments of the picture. The purpose is to deduce or to predict certain possible physiological or pathological states. With this approach, valid reasoning requires always the consideration of more than one kind of datum, and the speed of data acquisition becomes the paramount factor in drafting decisions leading to practical intervention, rather than conclusions having only a past interest.

The character of urgency in this quest for faster acquisition of laboratory data derives also from the prevalent economic, social, and demographic conditions, as documented elsewhere.<sup>2</sup> Tremendous problems now pervade the fields of public health, pathogenesis, environmental human physiology, disease control and preventive medicine, genetics, geriatrics and rehabilitation, and food production, distribution, and utilization. Such problems arise on a worldwide scale in the midst of rapid social transformations and with generally expanding national economies and growing populations. They create vexing enigmas that no government nor any individual can ignore. It is evident that their solution requires factual data of a specific nature based on a time scale that has become extraordinarily compressed by progress in the technology of communication and transportation. No doubt, it is only through a very complete automation of the processes of acquiring scientific data that one can expect to cope with these problems within a generation.<sup>6</sup>

The design and construction of automatic biomedical laboratory equipment must take into consideration other aspects of automation. The scope of operations must be the object of a complete system-engineering analysis.<sup>7</sup> For this purpose it is convenient to consider the functions of a biomedical analytical laboratory as an industrial operation capable of automatic performance once the proper sequence of function is mapped. Basically, industrial automation involves the accumulation of constraints so that all actions but the desired one are eliminated and "the control of machine by machines" becomes possible. The accumulation of constraints requires, not only the



application of an increasing quantity of machinery, but also adaptation of the production processes, a suitable end-product design, a convenient layout, and control of the input raw materials. Thus, in order to determine the best way of compounding these ingredients, it is necessary to determine the entire field's expansion, penetration, and density, to use the engineer's language. These aspects of the problems already have been developed at length in their relations to clinical laboratory operations and in their economic aspect.<sup>8</sup> The expansion factor refers to the abundance and diversity of data being sought. The most important aspect of this factor stems from the opportunities offered by scientific medicine to increase the life span, to reduce the incidence of disease, to promote rehabilitation, and to make old age productive. These opportunities, coupled with modern living and working conditions affecting millions in an unknown manner, make it imperative to review the long-accredited chemical and physiological "norms" on a broad population basis and with due regard for the ecological factors involved. The field depth penetration is concerned with analytical repetition in order to detect secular trends—variations in physiological norms brought about in the course of time—a rather new anthropological factor. The field-density factor scales the contemplated operations to the geopolitical requirements of the moment. Other factors, such as field span (the number of operation types to be coordinated), field level of complexity, and field coordination of secondary tasks such as data storage and diffusion are managerial aspects of laboratory automation that must be kept in mind for their potential impact on instrumental design.

Automatic analysis of biomedical materials also presents particular difficulties inherent in the nature of the specimens.<sup>4,9</sup> Such difficulties include (1) the small volume of fluid available, making the use of nondestructive methods mandatory; (2) the handling of numerous discrete specimens without confusing their identity during processing; (3) the frequent impossibility and usual inadvisability of multiple sampling; (4) the need to effect multiple analyses on a single specimen; and (5) the necessity of choosing the analytical program to be applied to each specimen on the basis of urgency and other extrascientific merits of the case, which often makes uniform programming impossible or, at best, uneconomical. Other difficulties arise from the cost or instability of certain reagents or the empiricism of many analytical procedures of proved practical value. This last factor, when present, defeats the aim of rigid standardization at each step of any fully automatic process.

The foregoing comments point to a number of characteristics desirable in an automatic analytical system: speed, reliability of components, ease of maintenance and of replacement, sustained accuracy over long periods of time, reasonable cost, incorporation of provisions for calibration and standardization and, above all, versatility.<sup>10</sup>

#### INSTRUMENTATION\*

##### *Choice of Analytical System*

A survey of biomedical laboratory techniques reveals that the most frequently applied procedures for research or clinical diagnostic purposes are, in

\* The equipment described in this section may be obtained from Ednalite Optical Co., Peekskill, N. Y.

order of frequency, colorimetry (including spectrophotometry), volumetry (including variants in which the end points are perceived either colorimetrically or electrometrically), and electrometry. Thus, two kinds of transducers should suffice to cover most needs, since considerable hybridization between several basic measurement methods are technically conceivable and some of these have, in fact, been reduced to practice.\* Radiation absorptiometry represents the bulk of all analytical requests encountered at present in this field. This situation may change in the near future, owing to the increasing emphasis placed on nondestructive procedures, for obvious reasons. In fact, electrometric methods, which usually require only one sampling operation, now are being extended rapidly for use in measurement of quantities other than  $pH$  and oxidation-reduction potentials.

These considerations led to the formulation of a program of investigation initiated in our laboratory in about 1954, which eventually branched out in several directions. These investigations included a study of various types of light transducers to determine their suitability in automatic electro-optical devices for biochemical measurement and recording,<sup>11,12</sup> electronic research with the aim of improving the design of photoelectric transducer amplifiers, and a search for a flexible programming method allowing complete freedom as to the sequence of analytical procedure to be performed, so that selection could be made randomly.

The equipment described below offers one workable solution to the last problem. The effort was centered upon the design of an improved primary measuring system, where most delay occurs at present. The transfer of information relative to the identity of the specimen through the analytical system is not considered at present, although it will become evident from the description that adequate provisions in the form of commercially available accessories for such transfer can be added to the apparatus without physical alterations. Similarly, the problems of data reduction, conversion, coding, printing, and storage can be solved by methods now well known and with accessories readily available. Consequently, the project could be limited to the designing of analogue read-out circuitry allowing eventual integration of the analytical system into a broader scheme of laboratory data and office automation. As a result of this policy, the analytical scheme adopted is capable of the following sequences of functions: sample metering, dilution and transfer, the metering and addition of reagents, control of reaction time and conditions, evaluation of reaction products, and data logging in analogue form.

The analytical apparatus includes three basic modules: a fluid-handling system, a measuring system, and a programming system coordinating the operation of the first two modules. These systems are described in sequence for semantic reasons. Actually, it was necessary to reach a decision regarding the measuring system before the other aspects of the project could be considered.

\* The Analmatic, manufactured by International Digitation Co., London, and other basically similar apparatus are examples of automatic intermittent titration or volumetric analysis devices in which the end point is perceived electrometrically whereas the reagent volume is read photoelectrically. Since they are automats simulating the chemist's actions, their programming system, which is of limited versatility, incorporates a rather complex network. See also page 673.

A time-motion study of the chemist performing a colorimetric measurement reveals the actual complexity of this routine operation. It is found to include, in the easiest case, at least fourteen steps to be effected in an invariable sequence (see R. Jonnard,<sup>8</sup> p. 166). The end result is a quantity called absorbance, from which a correction factor (blank) has been subtracted. The absorbancy is the analogue form of a chemical concentration.

In order to extend the scope of this preliminary investigation, it was decided that the system should be able to utilize more than one measuring transducer, so that a photoelectric-colorimetric measurement, representing the most complex operation, could be performed. Consequently, to test the practicality of this approach, provisions were incorporated into the programmer to accommodate electrometric measurements also. While the programming of two different transducers on the same read-out system was successful, the electrometric applications were not explored in sufficient detail. The examples of performance given herein involve only colorimetric measurements. It will become apparent that, in the present state of the art, other basic measurement methods such as refractometry<sup>13-15</sup> could be programmed just as conveniently. Consequently, the possibility of scanning several transducer inputs on a single data logging system acquires importance for its possible applications in the field of automatic industrial process control.

That all the measurements heretofore mentioned can be made continuously on a flowing stream determines the principle of operation adopted for the fluid-handling system. The entire system is based upon the principles of a continuous carrier fluid stream into which samples and reagents are injected in proper proportions in selected sequence and at the proper place. Reactions are allowed to take place in transit.

### *Normalization*

In the dynamic system contemplated, energy is necessarily imparted to the fluids handled. A pump is a convenient source capable of delivering energy at a constant rate. If the geometric dimensions of the system's lines are fixed, then duration and distance of propagation of the fluid are in finite ratio, and either one of the parameters can be used, depending upon convenience and the accuracy required. For instance, the length of a transmission line is a more convenient measure of a reaction's duration than is the elapsed time as measured by relays.

Similarly, sample and reagent volumes may be measured in terms of equivalent times, if the pumping rate is constant. Relative concentrations are thus replaced by relative rates of mass flow in lines of fixed geometric dimensions. In the final analysis, one deals with time-dependent variables, which can be controlled accurately by timers. This unique feature greatly simplifies the design of the programming system. Furthermore, the performance of the system now can be evaluated by the methods of frequency response analysis well accredited in chemical process and control engineering.

I had an opportunity to remark elsewhere (see Jonnard, page 160)<sup>55</sup> that the net effect of any measurement "results in the transfer of a certain amount of information in the direction of the observer," and that this transfer "could be done (by optical means) as well as by mechanical, acoustical or electric (elec-



tronic) means. In all cases the same differential equation, with suitable units, governs each system and incorporates the same terms (inertial, resistive, restoring function, driving function, response function, and response rate). The equation is that of the transient and the steady-state solution for a damped harmonic oscillator." This point is elaborated further subsequently in *Dynamic Performance*. The matter of harmonics generated in the measuring system greatly influences the design of any amplifier and the associated circuitry that may be required; hence, a relatively long discussion of amplification requirements is given in this paper.

The advantages of this approach to automatic analysis become invaluable features when the analyzer is used for industrial process monitoring, either directly or, in the case of complex processes involving several interrelated variables, through an intermediary computer. A full discussion of this point is, however, beyond the scope of this presentation.

### *The Fluid Processing System*

The success of the entire project depends upon the availability of small, inexpensive, accurate, yet adjustable pumps capable of delivering a metered flow at a very constant rate. A variety of pumping devices is known, all of which can be classified as either reciprocating or peristaltic. The reciprocating type is capable of metering fluids with a very high accuracy. The chief drawbacks are the mechanical complexity, the production of a pulsating outflow, and the need for occlusive devices such as valves. The peristaltic pump is appealing for its simplicity and the ease with which pressure fluctuations in the efferent stream can be smoothed out; furthermore, the continuity of the fluid channel in this device has considerable advantages for chemical and physiological applications. Positive occlusion by the rotating member eliminates the need for valves, a diaphragm, or similar components. Cleaning and maintenance are greatly simplified. Pumps for physiological investigations, in which controlled pulsations are reintroduced purposely<sup>16-18</sup> are categorized as peristaltic.

While peristaltic pumps offer definite advantages, from a general standpoint the fact remains that such devices, like other scientific instruments, are useful only within the context of the particular investigations for which they are designed. For this reason, it was necessary to build a pump specifically adapted to the problem at hand. A simplified design involving a minimum number of parts of simple geometry was achieved, so that the pump can be duplicated at low cost. The peristaltic action is produced by the motion of small rollers on a cylindrical rotor acting upon a length of Tygon tubing flattened against a semicircular runway. The necessary pressure is attained by adjusting a spring that allows the entire runway member a slight radial motion, thereby reducing wear of the tubing. The plastic tubing can be replaced without taking the pump apart. Tubings ranging from  $\frac{1}{32}$ - to  $\frac{1}{8}$ -inch inside diameter can be accommodated. Each pump carries a master gear, and its base plate allows a variable clearance between the gear and a horizontal shaft that provides a set of graded pinions. With this arrangement, a plurality of pumps can be geared to a common shaft and made to operate at different



relative speeds. Gear ratio adjustments coupled with a selection of suitable tubing diameters result in a fiftyfold range of available flow rates.

A minimum of one pump, one mixing unit, one gear shaft, and one motor is required to handle a given reagent. This constitutes the basic module. Rather complicated systems capable of simultaneously metering several fluids can be achieved by properly gearing several modules to a single motor, thus ensuring their simultaneous operation. The points of discharge of the mixers can be spaced with great diversity. Such an arrangement results in a great versatility in use.

Finally, an array of such systems can be assembled in series-parallel combination on a common base. By starting and stopping each system in proper sequence by means of electric timers it is possible to perform intricate chemical operations.

Control of reaction conditions and transfer time is among the functions of the fluid-handling system. Temperature control (cooling or heating) presents little difficulty; it is taken care of in a conventional manner, with thermostated enclosures disposed along the transfer line. The time allotted for temperature equilibrium is controlled by adjusting the length of line passing through a given control zone. The total reaction and transfer time between successive mixing units and to the measuring zone is adjusted similarly. In this way, time and conditions are controllable independently of the pumping rate or the proportioning operations. In all tests the fluids are processed in a completely enclosed system whose content is inaccessible to the operator. Thus, the chance of fortuitous contaminations is reduced, and maximum reproducibility of the result is more likely to be assured.

All the components of the systems are plug-in units or self-contained, readily interchangeable modules. One pump, mounted with a group of mixers, as described above, is shown in FIGURE 1.

If one anticipates the features of the measuring zone to be described later on, it is evident that this fluid-handling system can function also in a closed circuit. Thus, reaction rates can be measured and recorded, provided they do not exceed the shortest transfer time possible with a given setup, which depends on the time constant of the recorder, as will be explained. This feature opens up a large field of applications in enzymology.

With this arrangement, the apparatus reaction zone is rather indefinite, extending from the point of injection of the first reagent to the inlet of the measuring cell, and may be adjusted to meet extremely diversified requirements.

#### *Mixer*

A fast mixer for chemical kinetics studies has been described by Roughton and Millikan<sup>19</sup> and by Millikan,<sup>20</sup> utilizing a principle enounced earlier by Roughton and Hartridge. In this device, two fluids are ejected rapidly from motor-driven syringes in such a way as to meet at an angle of about 45° in a mixing chamber or a common duct of small diameter. It is easy to evaluate stream velocities (in cm./sec.) and hence the transit time at an observation point a fixed distance from the mixing point. Reaction times of the order of a

millisecond have been measured. Reaction progress has been followed in such streams with various instruments and techniques, among them the reversion spectroscope, thermocouples, photography, and photoelectric colorimetry with a capillary-sized observation zone (to reduce the time lag by speeding the fluid flow).



FIGURE 1. Reagent metering module. The unit includes a pump, synchronous motor, variable gear train, and one or several mixers.

The main difficulty is the blurring of the chemical front, due to the turbulent flow established at high velocity in narrow tubes. Indeed, the critical velocity at which turbulent flow starts is inversely proportional to the tube diameter. (In practice, the character of flow may be visualized by injecting a sharp, colored wave into the streaming effluent: as long as nonturbulent mass flow exists, this wave keeps its shape along the tube.) Obviously, the worst conditions for mixing exist when streamline flow occurs. Hence, two mutually exclusive conditions must be the object of a compromise.

Millikan's design is best suited to very fast chemical reactions, for the observation zone may be located extremely close to the mixing zone. A serious drawback, however, is the relatively large volumes of fluids required for efficient mixing, which depends upon a high rate of mass flow under turbulent conditions.

It is very desirable to attain efficient mixing at a relatively low flow rate, and independently of it. The lowest flow rate allowed is found from the value of Reynold's number; below this, flow is laminar and the effluent stream is considered to consist of an infinite number of concentric cylinders, each characterized by a different velocity, so that the contribution of each "differential cylinder" to the total mass transfer is different.

Thus, the practical problem is to produce and maintain turbulence at all flow rates.

In the general case, fluid flow obeys Poiseuille's law, so that the flow  $F$  is given by:

$$F = \pi r^4 dP / 8\eta l \quad (1)$$

where  $r$  is the diameter of the tube,  $l$  is its length,  $\eta$  the viscosity of the fluid, and  $dP$  the pressure drop along the distance  $l$ .

If the flow is laminar, one finds that  $dP$  is related to the velocity  $v_x$  of the fluid in any given differential flow tube of radius  $x$  (and cross-section  $2\pi x dx$ ) by the relation:

$$dP = v_x \cdot 4\eta l \cdot (r^2 - x^2) \quad (2)$$

EQUATION 2 shows that  $v_x$  may be decreased by increasing either  $l$  or  $1/dP$  or  $r$ . If  $dP$  is fixed, as it generally is, then by increasing  $r$  one may consider lower values of  $l$ .

Substitution of the value of  $dP$  from EQUATION 2 into EQUATION 1 gives:

$$F = \pi r^4 v_x \cdot 4\eta l \cdot (r^2 - x^2) \cdot \frac{1}{8\eta l}$$

or

$$= [\pi r^4 v_x \cdot (r^2 - x^2)] \cdot \frac{1}{2} \quad (3)$$

showing that a given flow  $F$ , being independent of  $\eta$  and of  $l$ , can be obtained by various combinations of  $v_x$  and  $r$ . If now  $r$  is made to increase progressively from beginning to end of the section  $l$  (the gradient of  $P$  along  $l$  becoming non-linear), it is evident that the same rate of flow  $F$  may be obtained with very many different combinations of  $\eta$ ,  $2\pi x dx$ , and  $dP$ , realizable within a given distance  $l$ . In each case the radial distribution of the cross-sections  $2\pi x dx$  of equal increment depends on the value of the stream velocity  $v_x$  at the input.

If one now considers two fluid streams arriving coaxially and with different input velocities in a zone of length  $l$  and variable  $r$ , it is evident that two corresponding radial distribution systems of differential laminar tubes are produced in superimposition upon one another. Furthermore, since the initial  $v_x$  values are different for the two systems, their rates of longitudinal propagation in a direction of increasing  $r$  remain different, and their trajectories must cross one another within the prescribed distance  $l$ ; perfect mixing of all the differential

flow tubes must occur, even if each stream is propelled so slowly that only laminar flow is possible.

The method should be applicable also with heterogenous fluids that seldom obey Poiseuille's law but, instead, tend to behave laminarily at *all* rates of flow.

It has been possible to ascertain the correctness of this reasoning by building a thin plaster-cast scale model or potential field analogue mapper according to Moore's technique.<sup>21</sup> Incidentally, a similar hydrodynamic model of the measuring cuvette described hereafter is often extremely useful in solving particular difficulties that may arise in the correct application of the Sampling Theorem with some chemical systems.

The exact performance of the mixer can be visualized by use of the indicator method described by Rossi and his associates.<sup>22</sup> The mathematical evaluation used by these authors, however, was found insufficient for the very low value of  $l$  under consideration. In practice, colored indicators were used to gather convincing evidence that complete mixing occurs at the exit of the mixer (about 20 mm. from the injection point), even at initial differential flow rates as low as 0.2 ml./min.

The mixing unit consists of a molded nylon body with two input connections. The two fluids to be used are made to flow coaxially into a common flared nozzle of carefully measured taper angle cut out on the outlet side of the body. The total hold-up volume is about 0.2 ml. The flared nozzle is only 10 mm. long. The whole unit is 50 mm. long and 25 mm. in diameter.

### *The Sampler*

The functions of the sampler are (1) metering and then transferring of the sample into the analytical zone of the system with or without dilution as required, and (2) uniform propulsion of an uninterrupted carrier fluid stream.

The sampler consists of a rocker arm actuated, at present, by a 40-rpm electric motor, a magnetic clutch, and an eccentric cam. The motor runs continuously at constant speed. The rocker-arm motion, controlled only by the fast action of the clutch, thus is subjected to much shorter accelerations and decelerations than would be possible if the effect of the inertia of the motor were not eliminated. The arm carries a capillary sampling tube that, at the lowest point of its course, dips into the analytical specimens presented in suitable containers. The sampling tube is connected through a tee-connector to one peristaltic pump in continuous operation. The other side of the tee-connector is attached to a reservoir containing the carrier fluid, usually water. Simultaneously, the arm operates two spring-loaded rods that crush alternately the plastic line to the reservoir when the rocker arm goes down and the sampling line when the arm rises outside the specimen container. In this way, a column of specimen of finite length and hence, of finite volume, flows uninterruptedly through the system between two sections of the carrier fluid. It is possible to prevent the entry of air bubbles at the sampling tube opening by properly adjusting the spring-loaded rods. The holdup in the sampling tube is of the order of 0.010 ml. The resulting contamination of a subsequent specimen is usually negligible. When this error must be reduced further, a new adjustment of the rods can be achieved whereby a small air bubble is



admitted into the system. With most chemical reactions the presence of small bubbles has no effect upon the result.

FIGURE 2 is a front view of the sampler, showing the eccentric cam, the rocker arm, the adjustable rods, and one reagent pump. It is convenient to locate the first reagent pump immediately next to the sampler in order to reduce the dead space. At the outlet of the pump it is necessary to interpose a mixing

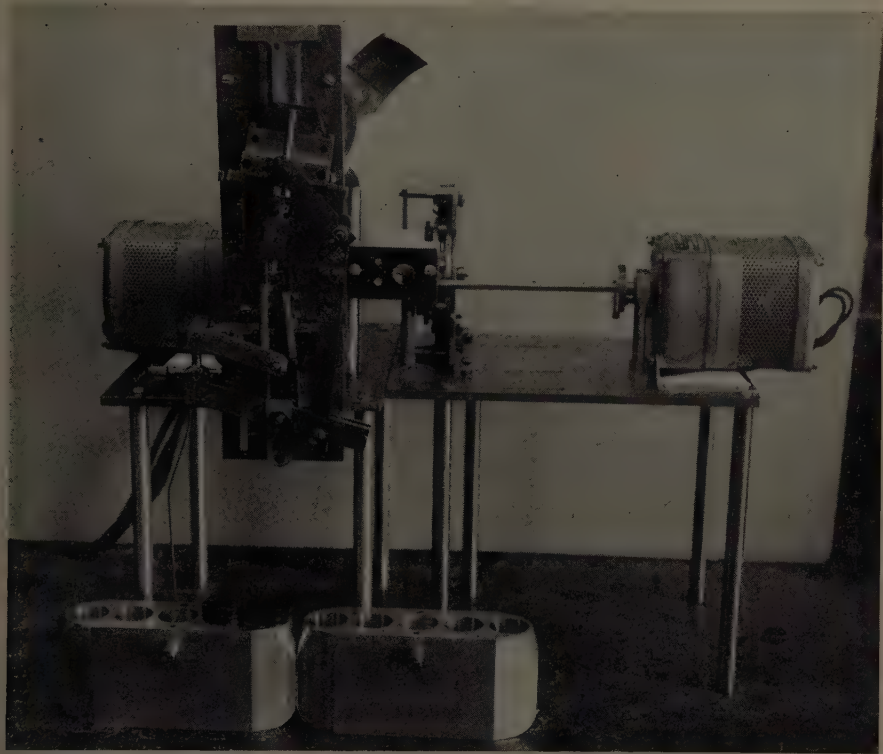


FIGURE 2. Sampler, front view, showing the rocker arm, sampling tube, eccentric cam, one mixer, and one reagent pump.

unit. A side view of the sampler motor proper, the sampler pump, and the clutch is illustrated in FIGURE 3.

Exact metering of the sample is achieved by controlling the time allowed the sampling tube to dip into the specimen. This control is achieved by means of an electric cam timer acting on the sampler magnetic clutch. Such a timer is relatively inexpensive. It is possible to have a battery of cams on such a timer, each set permanently for each chemical procedure that the system is capable of carrying out. Indeed, any procedure, regardless of its complexity, never involves more than one sampling operation. The sampler multiple-cam timer is located in the program unit to be described.

In addition to the sampler timing control, the reciprocating action of the

eccentric cam lends itself conveniently to adjustment of the sampling tube stroke. An extremely wide range of sample volumes can be realized by combining this feature with the proper adjustment of the timers. Furthermore, the stroke at the eccentric cam can be regulated during operation, whereas the resetting of the timer's cams is a lengthy procedure. These features, invaluable

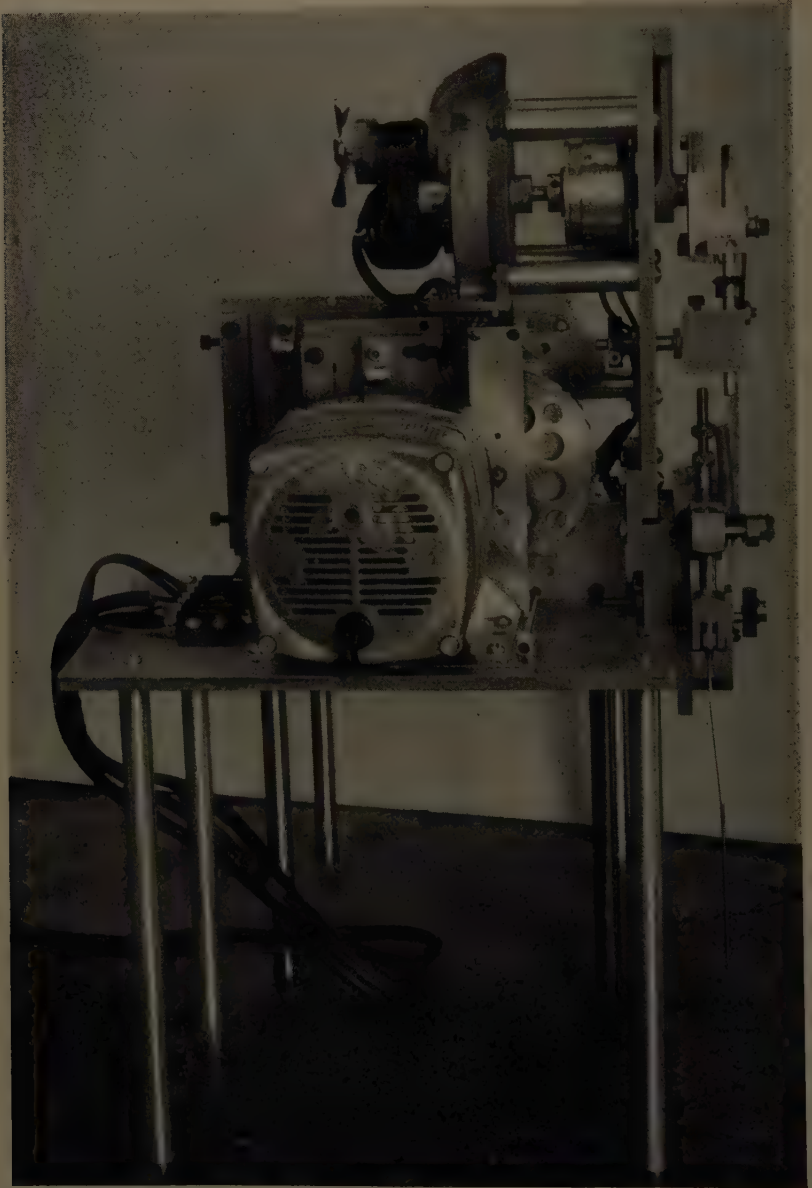


FIGURE 3. Sampler, lateral view, showing the sampling pump and motor, eccentric cam, motor, and magnetic clutch.

during the developmental phase of our work, probably would be unnecessary in a commercially designed unit. It is also realized that greater simplicity and economy would result from the use of linear motions controlled by reciprocating solenoids, which would eliminate at least one motor and the clutch from the module.

### *Choice of Measuring System*

Some of the requirements specified for the fluid-processing system apply equally well to the measuring system, particularly those physical factors influencing the transfer function (equation 6). For practical reasons, it was necessary to reach final decisions regarding the operative mode of the measuring system adopted before developing the programmer.

The measuring system consists essentially of a measuring zone through which the reaction products flow continuously past the measuring transducers, plus a unique amplifier followed by an analogue data logger (at present, a modified strip-chart recording potentiometer).

The present measuring system can receive signals from different kinds of transducers located in the measuring zone. Provisions must be made in the programmer for serial presentation of these inputs. Furthermore, since the modular design of the fluid-processing system allows for operation of several reaction channels in parallel, it is conceivable that a plurality of either identical or different transducers could be placed at various locations in the apparatus and, in all cases, programmed serially at the logger input. Thus, the measuring zone is not limited to a single geometrically defined enclosure but extends over a plurality of discrete, specialized transducer locations.

In the present state of development, the apparatus can utilize either photoelectric signals, electrochemical cell potentials, external calibration voltages, or pressure transducer signals for certain physical measurements.

It is noted in the description of the programmer that the process timers select simultaneously the proper input connection to the transducer required for the particular analytical process chosen. The wiring diagram (FIGURE 8) shows one of the operating circuits, each of which requires the closing of a single contact. In practice, the actual connection to the transducers is made indirectly, through an intermediary stepswitch and a ratchet acting on a silver contactor of the enclosed type. This entire mechanism is built as a module and shielded completely in a hi-mu metal box.

Automatic data logging and reduction require that the read-out device scans rapidly the various incoming signals. The input impedance of the device must be adaptable to a rather large range of internal resistances of signal sources. If the system is to remain calibrated over sufficiently long periods of time, the amplifier input impedance must be higher than the resistance of any of the transducers used. This difficult objective can be achieved by suitable design of the amplifier and/or use of any of several methods of feedback.

Decisions regarding the final design of the circuit required an exact evaluation of the joint performance of the transducers and the fluid-processing system.

### *The Colorimeter*

Simplicity and reliability were the paramount factors considered in the selection of the colorimetric device. Other characteristics considered important

were the short-term and long-term photoelectric stability, the precision desired, the zero-stability of the circuit, and the spectral sensitivity of the light transducer utilized.

The problems involved in the selection of a basic colorimeter and its redesign or adaptation to the automatic measurements were solved in a somewhat empirical manner, but it is useful to summarize the arguments, the issues of which are often beclouded by a regrettably confused terminology.

The terminology used herein follows as closely as possible the recommendations of the Committee on Colorimetry of the Optical Society of America,<sup>23</sup> the American Society for Testing Materials, the National Bureau of Standards Circular of 1947,<sup>24</sup> the American Standards Association,<sup>25</sup> and the American Chemical Society's Joint Committee on Nomenclature in Applied Spectroscopy.<sup>26</sup>

The definitions and symbols are those generally accepted in contemporary technical literature.<sup>27</sup> However, the symbols  $P_0$  and  $P$ , representing respectively the incident and emergent radiant power flux (in a finite solid angle defined by the absorption cell), were deleted, since the determination of these quantities requires rather involved measurements. Instead, the more universally used symbols  $I_0$  and  $I$  were retained, since the fractions of  $P_0$  and  $P$  attributable to reflectivity and refractivity are in constant ratio whenever the refraction error can be neglected (as in the case of measuring cuvettes with parallel faces), as shown by Reilley and Crawford<sup>28</sup> and by Crawford.<sup>29</sup> Indeed, it must be kept in mind that radiation transducers and phototubes or photronic cells connected to galvanometers as ordinarily used actually measure, as does the eye, the radiant flux (or time rate of flow of radiant energy) in a given solid angle, that is, the radiant intensity. These transducers are incapable of time integration, although they are capable of spatial (or spectral) integration. In compliance with the above conventions, the following symbols are used throughout:

- $T$  per cent transmittance of a given sample, referred to incident  $I_0 = 100$ .
- $T_s$  per cent transmittancy of a solution relative to its solvent or to a blank.
- $A$  absorbance, equal to  $-\log T$  (originally, optical density).
- $A_s$  absorbancy, equal to  $-\log T_s$ .
- $E$  molecular absorbancy index, equal to  $A_s/bc$ .  $E$  is equal to  $k$ , the extinction coefficient, when  $b$  is the thickness in cm. and  $c$  is the chemical concentration in moles/l. at a stated temperature (Gibson<sup>30</sup>).

A barrier-type or self-emissive phototransducer was selected to satisfy the requirements of simplicity and reliability. The intrinsic qualities of a given instrument can be conveniently considered independently of their effect upon the measurements ultimately made with it, since measurement precision always is influenced greatly by methodological errors superimposed upon the instrument's limitations.

Assuming that a *constant-emission* light source is realized, the choice between a single receptor and a compensated type of instrument can be made solely on the basis of the properties of the photoelement. A judicious evaluation of this problem must depart from the oversimplified statements in most textbooks, according to which (1) the output voltage  $V$  and the output current  $I$  are related by the simple relation  $V = AI^p$  (where  $p \approx 0.4$  and  $A$  is a constant),



and (2)  $I$  varies linearly with incident light intensity when the external resistance is *sufficiently* low. Unfortunately, things are not so simple—our idea of the inner functioning of such receptors is still nebulous. A more satisfactory, albeit empirical, relationship between  $I$ ,  $V$ , and the internal and external circuit conductances was developed by Romain.<sup>31</sup> The exponential formula given is difficult to manage and must be developed into a converging series. One thus discovers that the external circuit resistance, even if kept low, remains an important factor whose relative influence depends on the level of illumination. This factor must be taken into consideration if the electric measurement error is to be kept below 1 per cent. Such precision is not excessive, in view of the fact that a 1 per cent error in transmittance corresponds to a 2.8 per cent error in chemical concentration.

Details of the mathematical treatment aside, the serial development mentioned above indicates that it is almost impossible to construct a measuring system of the required accuracy that is linear with light intensity, by balancing differentially two transducers on either a potentiometer or a bridge circuit. Differential aging of the transducers adds to the difficulty and excludes permanent calibration. One grave objection to so-called balanced circuits is their decreased sensitivity near the balance point, where sensitivity is needed most. Even the commonly accepted statement (Summerson<sup>32</sup>) that the use of a balanced circuit minimizes the effect of light source fluctuations is fallacious. The serial treatment shows that, in reality, the "reference current" in the second photoreceptor varies as the fourth power of the line voltage, and that approximate compensation is possible only at the 100 point on the scale—where it is not needed—and only for one level of illumination at a time.

For these reasons, the Rouy single cell, clinical colorimeter was adopted as the basic measuring device.

The extreme reproducibility of the photoelectric current is maintained only if the transducer is handled properly. Commonly used impinging light intensities of approximately 50 ft-c are much too high, and temperature effect *within* the photosensitive layer must be avoided. Forced ventilation was found sufficient without recourse to controlled cooling of the transducer if the light was kept below  $\frac{1}{100}$ , and preferably below  $\frac{1}{1000}$ , of the level given above. Under these conditions the tails of the well-known spectral sensitivity curve for photronic cells extend so far into the UV and IR domains that measurements can be made from 270 to about 1000  $m\mu$ , in the absence of the large sensitivity peak in the visible spectrum, provided that adequately sensitive means of measuring the low photocurrent are available. Sufficient flattening of the spectral response curve is obtained with the peak-selecting filters supplied. Single sharp cut-off filters are not usable, and interference filters and wedges do not allow long-term calibration.

In order to take full advantage of the broad spectral range available, the 6-v. lamp of the colorimeter is mounted interchangeably with a 4-w. mercury insecticidal tube in a separate housing (visible in FIGURE 15).

Although the sources of light are powered through voltage-regulating transformers, it is essential to ground physically the case of the instrument and the cores of the transformers directly to the building frame. In addition, a shielded three-wire input connecting cable must be provided, connecting its ground lead

(red) to the "negative" side of the measuring circuit, directly at the photoelectric cell terminal. No other grounding lead should be allowed in the *entire* apparatus, to avoid "ground loops."

It is convenient to connect the transducer to the read-out system (*v.i.*) through an attenuator network incorporating a multiplier circuit (FIGURE 4). The entire network must be fully shielded. A bucking current is derived directly from a small tap on the transformer powering the colorimeter lamp and is fully rectified (and filtered) so that its eventual long-term fluctuations remain in phase with those of the power line. Calibration signals are obtained

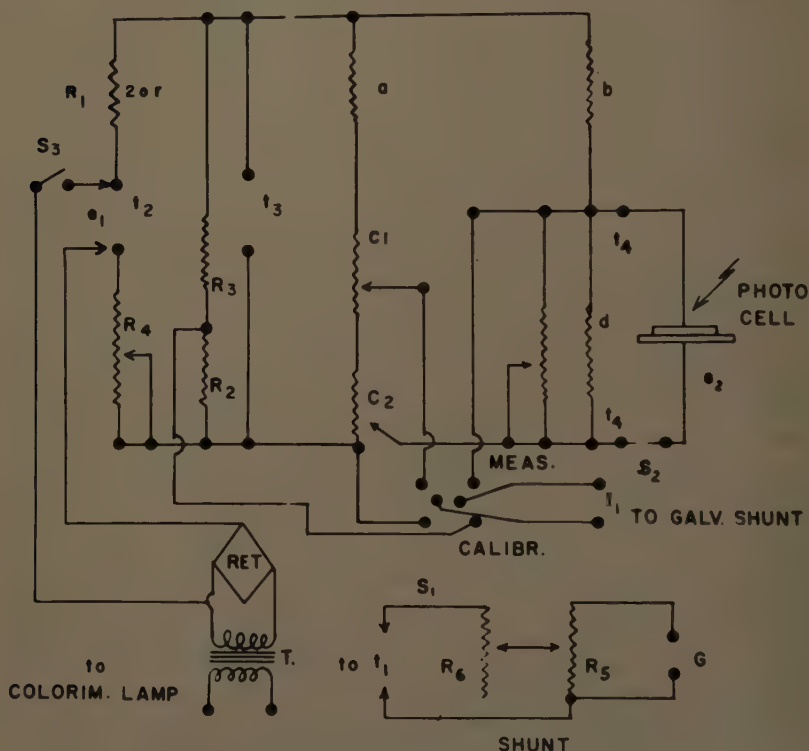


FIGURE 4. Barrier-type photoelement compensation network.

from this auxiliary circuit. Similarly, zero-shift or scale-expansion techniques of measurement may be employed, provided the photonic cell is *never* subjected to a bucking potential larger than the photopotential to be measured (that is, the output current should *never* be reversed), lest its sensitivity and stability be greatly impaired for many hours subsequently. Thus, with the proper precaution, the effective range of absorbance measurements may be extended by several logarithmic periods to include optical densities considerably greater than 2.0.

In the circuit shown, potentiometers  $C_1$  and  $C_2$  are available for coarse and fine manual adjustment of the scale span.

The potentiometer shown in parallel with resistor  $d$  in reality comprises a

set of 10 trimmers connected to the automatic sensitivity selector switch of the programmer, so that the scale span automatically is set correctly for each analytical procedure. Occasional departure from this setting, occurring when the reference solution (water, blank, or other) is presented to the instrument, can be corrected readily by operator adjustment of  $C_1$  and  $C_2$  as usual.

All precautions are taken to avoid generation of thermal emf in the measuring circuit. This source of error often amounts to 4 or 5 per cent of the measured transmittance in some commercial colorimeters.

The temperature dependence of the photronic transducer is less generally appreciated. The response of a phototransducer depends only upon the extent of the area common to its spectral sensitivity curve and the emissivity curve of the source. If the full spectrum of radiation from a hot source is utilized, the black-body temperature of the source is the predominant factor. When a luminescent source (a UV lamp) is employed, however, fluctuations in ambient room temperature may have a predominating influence. With most photronic cells, the common area at  $800\text{ m}\mu$  is about 1 per cent of the maximum measured at  $500\text{ m}\mu$  with the source at a black-body temperature of  $1400^\circ\text{K}$ . Since the common area varies as the tenth power of the source temperature, according to Larsen and Shenk,<sup>33</sup> rather large errors may result from small variations of source temperature. As the source temperature drops, the transducer behaves progressively more like a pyrometer whose response  $E$  versus absolute temperature is approximately of the form  $E = kT^{12.34}$  (that is, the plot of  $\log T$  versus  $E$  is a straight line). Accordingly, underrating the source results in considerably decreasing the relative response to thermal radiation, thus indirectly improving the response stability to selected radiation in the measured range, provided sufficient amplification (or sensitivity) is available.

Other features relative to the color filter system and the measuring cell are discussed in subsequent paragraphs.

The lowest limit of radiant flux utilizable is determined by the reversal potential of the barrier-type photocells. This potential I observed more than twenty years ago (unpublished observations). It destroys the response linearity of the transducer, and may introduce an enormous error into measurements with strongly absorbing fluids. This reversal, which exists in various degrees at all wave lengths, probably could account for certain difficulties in interpreting numerous colorimetric determinations reported in the biochemical literature (Jonnard<sup>34</sup>). For low radiant flux measurements it is essential to provide means of correcting this in the measuring circuit, in the form of a constant positive factor or upward scale shift voltage. Referring to a figure given in a previous publication (Jonnard,<sup>8</sup> FIGURE 5), it is apparent that the correction is possible only if the amplifier is insensitive to the phase of the input signal. Hence, an important new requirement is placed upon the amplifier design.

### *Continuous-Flow Measuring Cell*

The design of the compartment defining the volume of liquid being observed for measurements is still one of the major factors governing colorimetric accuracy.

In a continuous-flow instrument of the single-receptor, single-beam type, the

only geometric requirement is that the thickness  $l$  of the fluid layer remain constant.

In order to avoid the variable refraction error, which is always large with cylindrical cuvettes, a square-section cell with plane parallel faces and a thickness close to 10 mm. is used. Holdup must be reduced to a minimum if reasonably sharp chemical fronts are to be detected in a flowing stream. Consequently, the internal width is reduced to 7 mm. Finally, flushing of the cell between determinations, which is a lengthy operation with certain solutions, is facilitated considerably by building the body out of a nonwetttable plastic material such as nylon. FIGURE 5 illustrates the construction of such a nylon cell, one designed to be equipped with stress-free removable quartz windows. The cell fits into the well of the clinical Rouy colorimeter. The two vent tubes located at the inlet and outlet, respectively, prevent air bubbles from reaching the measuring compartment. The direction of flow within this compartment is upward.

For nearly two years of continuous operation, this cell has proved satisfactory but not entirely trouble-free. Nylon is not quite as inert to common chemical solutions such as dilute alkalis and iodine as is often claimed. The formation of microscopic gas bubbles on the windows due to the action of oxidizing reagents such as dilute nitric acid, dichromate, and molybdate is occasionally a problem. While other designs are still being investigated, notably a whole quartz construction, the present nylon model is offered for its proved performance and its general usefulness.

Optically flat inserts can be used when it is desirable, as in reaction kinetics studies, to reduce further the hold-up volume and to sharpen the colored front.

The plastic body construction is suitable for combined measurement of color and  $pH$ ; commercial capillary electrodes can be inserted easily without interfering with the light propagation. A convenient design for such a purpose was proposed by Boaz and Forbes.<sup>35</sup>

The use of a single cuvette of thickness  $l$  for *all* measurements, as reported here, sharply limits the error on chemical concentrations. Assuming a constant incident light intensity, a differentiation of the modified Beer-Lambert law

$$A_s = -\log T_s = klc$$

yields

$$kc \, dl + kl \, dc = 0,$$

showing that the relative percentile errors due to  $c$  and  $l$  are equal but of opposite sign. The final error evidently is minimized if  $l$  is made constant for all determinations, including blanks, standards, and the reference solution used to define the scale span (water,  $A_s = 0$ ).

Further improvements in accuracy can be achieved in some cases by a modification of a technique known as the "transmittance ratio method." Ideally, the concentration error is minimum when the transmittances of both unknown and standard solutions are almost equal. The method of Jones *et al.*<sup>36</sup> can



be applied with the present system, requiring merely the addition of one proportioning pumping system to readjust the concentration of the standard solution until it becomes equal to that of the unknown. The limiting factor then becomes the precision of the titration of the standard, and the resulting

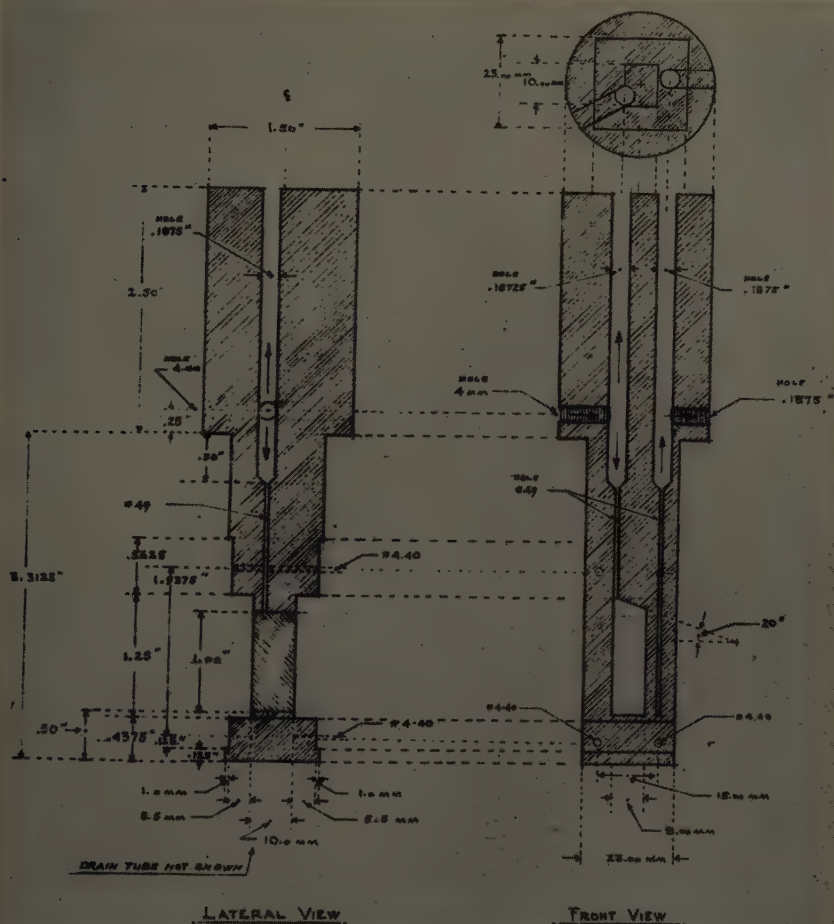


FIGURE 5. Automatic colorimeter with continuous flow cell (nylon construction).

reduction of error on the concentration remains small. The advantages of the transmittance ratio method were assessed fully by Gridgeman.<sup>37</sup> They are best ensured when a single cuvette is used for all measurements involved.

### Colored Filters System

The set of colored filters supplied by the manufacturer was found generally satisfactory. Others may be added for specific purposes.

The instrument remains a finite band-pass measuring device in which integration of the distributed energy is achieved photoelectrically. The method of integration, considered from this standpoint, is valid provided the band width of the filter utilized remains somewhat smaller than the half-band width of spectral absorption of the liquid being analyzed. Such a choice of conditions, while not theoretically perfect, results in a practically satisfactory compromise between the operating factors that can be deduced from the equations published by Strong.<sup>38</sup> It is particularly applicable whenever the photoelectric spectral sensitivity curve is approximately flat within the spectral domain considered, and when the reference fluid resembles closely the unknown (transmittance ratio method of measurement).

For automatic operation, the circular filter holder is operated by a 10-position solenoid selector of conventional design.\* Thus, a single pulse produced by the closing of a contact on the program keyboard simultaneously determines the fluid-handling schedule for a given analytical procedure and the wave length of the filter to be used for the final measurement. Another wafer of contacts on the same selector connects the proper shunt trimmer or attenuator (see FIGURE 4), so that the upper end of the read-out scale corresponds to the transmittance of either the blank or the reference fluid to be used conjointly with the procedure in progress or some fraction thereof, if one of the more sophisticated methods of transmittance ratio measurements is employed.

Reliability of operation is ensured by the mechanical linkage thus realized between the colored filter selector and the input attenuation resistor.

The wiring of the colorimeter automatic setting mechanism is illustrated in FIGURE 6.

The double-pole, double-throw (DPDT) galvanometer switch is used merely for occasional monitoring of the colorimeter input against a reference instrument of known sensitivity (50  $\mu$ a full scale, 150 ohms internal resistance). The signal-carrying lines, including the attenuator rotary contact coupled to the color filter wheel, are shielded completely from stray potentials between the photocell and the recorder input connections. The dotted line from the Leland stepper front wafer to the filter holder represents the mechanical linkage between these 2 components. The remote programming keys are illustrated for demonstration purposes. It is clear that the setting of the measuring system is fully determined by the temporary closing of any one of the contacts available. Actually, the contacts represented are operated by the process program timers, so that the positioning action does not occur until just a few moments before a given analytical solution reaches the measuring zone. Thus, the programming of any given procedure available in the apparatus can be initiated at any time without interfering with the completion of a previous analysis still in progress. The details of this time-sharing method will be clear after considering the details of the process programming circuit.

A safety circuit on the rectifier output line (not illustrated) further prevents the step switch solenoid from being energized if the previously programmed analysis has not yet been completed. Energizing of the solenoid takes place only after the recorder stylus has regressed from a previously recorded peak

\* G. H. Leland Mfg. Co., Dayton, Ohio.

reading by a distance (or time) sufficient to satisfy the minimum sampling time condition discussed under *Dynamic Performance* (EQUATION 10). Otherwise, the incoming analytical sample goes to waste and the recorder pointer returns to, and stays at, the level of zero absorbancy, indicating that the analytical system has not been programmed properly. Other embolic requirements are considered subsequently.

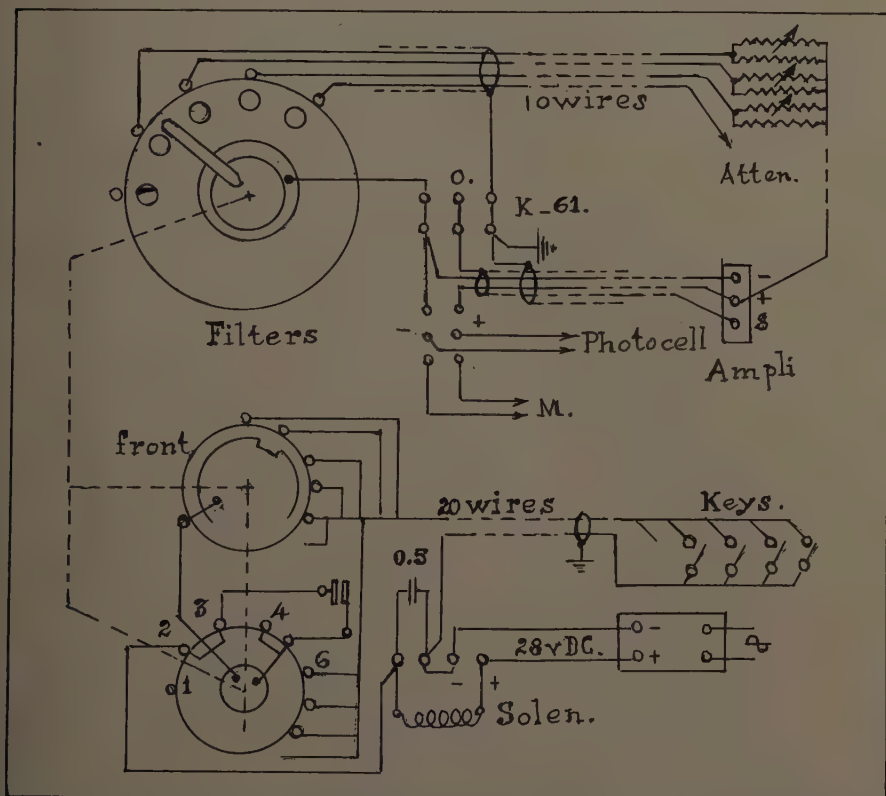


FIGURE 6. Automatic colorimeter programmer. The module includes the colorimeter filter wheel, input automatic potentiometer trimers, and one solenoid stepswitch (front and back control wafers only are illustrated). Control by key or cams remotely located on the process unit programmer (FIGURE 8).

### *The Sampler Programmer*

The functions of this programmer are to start the operation of the sampling device and to determine the sampling volume or the equivalent sampling time.

Economy of equipment is achieved by combining several components into this modular unit. The sample being metered must be propelled simultaneously toward a reaction or a measuring zone. Consequently, the carrier fluid pump is operated conjointly. However, it is preferable to stop this carrier stream during the actual sampling operation in order to avoid diluting the

sample. Further consideration of the subsequent treatment of the sample helps to simplify the instrumentation, even though this treatment is effected outside the sampling device proper.

The wiring diagram of the sampler programmer is given in FIGURE 7.

The heart of the device is, again, a lock and reset type of cam timer. The plug-in module incorporates one timer and a fully wired connecting strip. The operating contacts shown on the diagram may be located at a considerable

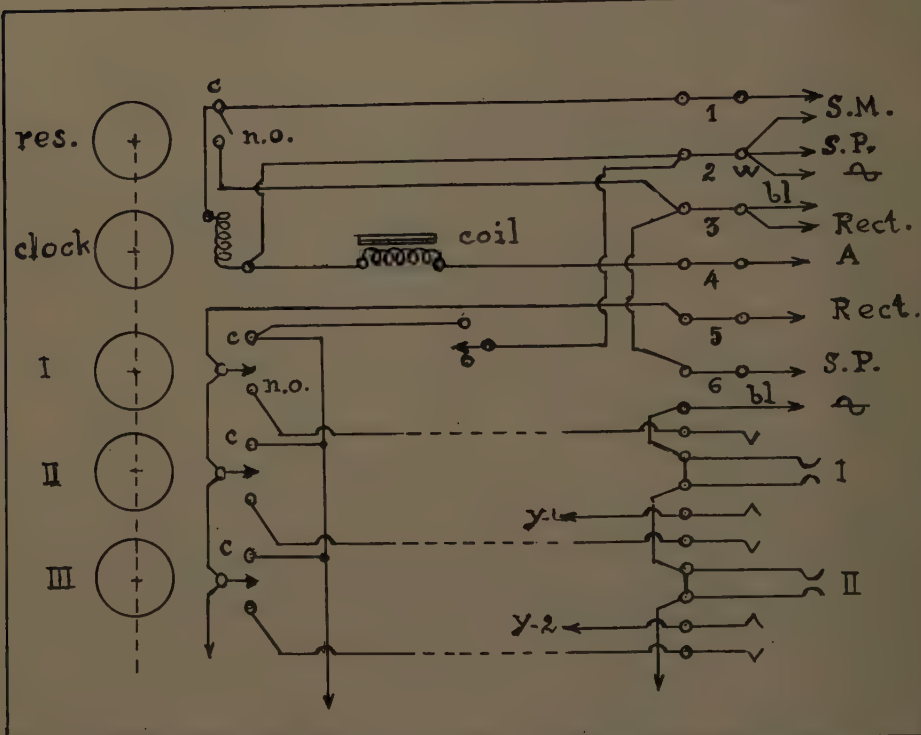


FIGURE 7. Sampler programmer. The module includes one cam timer (lock-and-reset type) and one connecting strip. The commanding keyboard (DPST push-button keys) is remotely located.

distance. It is immaterial whether these contacts are operated by means of manual keys or mechanical cams.

The number of active cams on this timer determines the number of finite sampling volumes available in the entire system. At present 10 different volumes are programmed. Certain commercial timers contain as many as 20 cams. It is also possible to operate a bank of several such timers in parallel on the same wiring strip and keyboard. In addition, sampling volumes are adjustable, as previously explained.

Operation of the sampling tube by means of a magnetic clutch results in a relatively complex wiring. The sampler motor, however, runs at full speed at all times.



The closing of a double-pole, single throw (DPST) contact on the keyboard results in a pulse being received by the chosen process programmer (Terminal 3, FIGURE 8). Simultaneously, the corresponding sampler timing cam is energized, but its contact is not operated. This contact closes upon positive command of the process timer (FIGURE 8), and this occurs only after the first reagent pump is set in motion. Simultaneously, the sampling pump is started. The sequence of operations is: (1) the keyboard starts the process timer, and (2) starts corresponding first reagent pump (if several reagents are required, the other pumps will then be actuated seriatim); (3) the process programmer sampler cam starts the sampler programmer motor and the sampling motor; (4) the sampler cam corresponding to the chosen process becomes energized a fraction of a second after step 3 and actuates the magnetic clutch, so that the sampling rocker arm dips into the specimen container for a measured length of time and then returns to its position of rest.

The sampler timer motor continues to run for one complete cycle, about 4 min. or the duration of the longest sampling operation to be performed by the system. Time sharing is possible whenever several analytical processes are operated in sequence and the sum of their sampling time is smaller than the duration of one full cycle. With such a sampling program, the process line contains the following succession of fluids: the first reagent—a short column of carrier fluid—the first reagent diluted in finite proportion with the carrier fluid (blank)—the first reagent diluted in the same proportion by the unknown sample—and a column of the same reagent diluted by the carrier fluid. At the end of the operations the entire vein of fluids is propelled by a column of the carrier fluid alone, and is made to progress forward until the measuring system has completed its read-out cycle. In the meantime, other reagents can be injected along the transfer line by the action of other cams on the process programmer, so that relatively complex reactions can be effected.

### *The Process Programmers*

A simple process programmer consists of an assembly of one cam timer and one completely wired terminal board. One such unit is required for each analytical procedure available in the apparatus. The timer, of the lock and reset type, includes one reset cam, one sampling cam, one transducer-setting cam, and as many more cams as there are reagent pumps to be activated. The sequence of operations in any analysis is always fixed: sample metering, reagents metering and addition in the proper order, reaction mixture transfer, and measurement of some selected property such as pH or color after a definite time. Such sequence of operations as may be required is rigidly controlled by setting the timer's cams relative to one another. The wiring of one unit, requiring only one reagent pump for the sake of clarity, is illustrated in FIGURE 8.

These plug-in units connect directly to the 115 v. AC power line, with due regard to the location of the neutral line terminal. One line (marked A) from each of these units is common to the sampler control timer (FIGURE 7). The entire unit is energized by closure of a contact connecting at point 3 of its terminal board. The remainder of this control circuit is shown in FIGURE 6. In this way the selection of the chemical process to be effected determines the

sampling time, and hence the sampling volume, as well as the start of the sampling cycle. After an energizing pulse (one twentieth of a second) is received at terminal 3, the entire sequence of operations scheduled into the chosen programmer unit continues by itself. The main programmer keyboard is again available immediately after completion of the sampling cycle, although the analysis just initiated is far from finished. Pilot lights (not shown) are wired to indicate at all times which analytical system is in operation. The front and the rear views of the entire programming unit are illustrated in

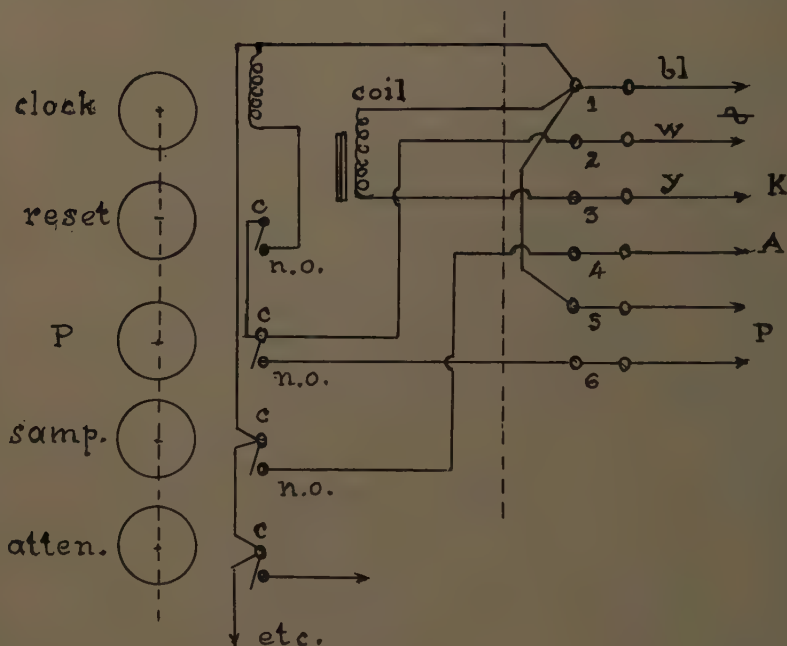


FIGURE 8. Process unit programmer. The module includes one cam timer (lock-and-reset type) and connecting strips. The filter-selector cam actuates the colorimeter filter wheel. The same cam is used for the selection of input measuring transducer.

FIGURE 9, right and left, respectively. The system is contained in a table-mounted radio cabinet of standard size for 19-inch panels.

The remaining minor aspects of the programming problem refer more particularly to the conversion of the data to a form suitable for ultimate calculation of chemical concentrations. These are discussed briefly along with the recorder.

#### *Automatic Titration*

Although it was not initially intended to explore the field of titration, some recent experiments proved that the equipment described can be converted easily to perform automatic volumetric analyses with either colorimetric or electrometric end points.



FIGURE 9. Programmer. *Left*: rear view, *right* front view.

For this purpose, the sample is metered as usual and the system is realigned for closed-circuit operation. The titrant then is pumped slowly into the circuit at a constant rate until the end point is reached, when the recorder usually indicates an unambiguous change of deflection. To measure the titrant, a 3-v. battery and potentiometer are geared down to the titrant's pump shaft, so that the duration of the titration and hence the volume of titrant are converted into a proportional voltage. It is a simple matter to translate the recorder deflection change into a signal that, by means of a micro-switch, now allows the recorder to print the volume of reagent used up at the same time it stops the metering pump. The potentiometer must be calibrated volumetrically for each reagent. Satisfactory performance is due chiefly to the efficiency of the mixers described; this results in a considerable economy of equipment over that used in other volumetric systems described in the chemical literature.<sup>39</sup>

#### *Internal Check-out: Embolic Requirements*

In a system as complex as that described, there appears to be a need for automatic internal check-out and malfunction diagnosis, if reliability is to be maintained in continuous operation.

An ideal check-out system should be reliable, versatile, automatic, and easy to maintain, and it should document its action with a record. In this particular instance it is also desirable that such a system perform periodic analytical control operations or calibration tests. At present, many of the checkout and diagnostic tests are accomplished by means of pilot lights that present information to the operator, who then makes appropriate decisions. It is conceivable that eventually an independent, automatic checkout unit will be attached to the main analyzer, thus considerably enhancing its dependability (Lauler *et al.*<sup>40</sup>). A simplification of the task involved is expected from the use of a plurality of identical components or units that can be assembled in modules of variable size and complexity to constitute the rather versatile analytical apparatus described herein. (Detailed comments on the problem of reliability in machines and on its evaluation are given elsewhere.<sup>41</sup>)

At present, interlocking circuits with flashing lights are provided to give notice of failure of the colorimeter exciter lamp, the cooling blower, the various rectifiers and solenoids of the stepping switches, and various components of the amplifier and the recorder.

A check of the scale zero also must be obtained, as previously mentioned. In the actual running of identical serial analyses by one of the dynamic procedures mentioned, it is quite desirable to obtain a blank, or a reagent check point, for every ten tests. In addition, the scale span is checked by an interlocking circuit that prevents operation of the recorder if the pointer should not return to the top of the scale ( $A_s = 0$ ) at least once between two successive but dissimilar analyses. Of course, more complex electronic methods for automatic control of scale span and sensitivity revealed recently in the technical literature could be incorporated easily into the system described without altering its hydrodynamic performance.



*The Amplifier*

It was pointed out previously that solution of the general problem of scanning a single read-out system over a plurality of measuring transducers requires an impedance-matching amplifier. Such an amplifier must perform the necessary input-output impedance matching for each transducer. In the present case, it must provide in addition fairly large amplification of the photoelectric signals. Indeed, the specifications listed for correct use of barrier-type photoelements for continuous recording over an extended spectral range—flattening of the spectral sensitivity curve by heavy selective filtration and low radiant flux—coupled with the low emissivity of most radiation sources outside the visible spectrum, results in signals in the millimicroampere ( $m\mu a$ ) range. Moreover, amplification is always required for electrometric measurements.

Several years' research has resulted in an amplifier design that is a compromise between the conflicting requirements of different kinds of input transducers. Particular attention has been given also to the matter of short- and long-term drift.

The importance of reducing instrumental drift in automatic analysis cannot be overstressed. It is often assumed that this error is minimized in differential procedures in which an unknown and a reference solution are compared either simultaneously or in quick succession. When the series of random measurements is small and spans a relatively short time, a consideration of the individual differences between duplicates, or of their averaged squares cannot reveal the extent of the drift error. The same applies, to a lesser extent, to the standard deviation when a small series of random measurements spanning a relatively short time is performed without the benefit of preliminary statistical planning—as too often happens. Under such circumstances, there is always the danger of recording results incorporating a consistent error.

The only alternative is prior statistical planning and experimental verification that the calculated drift is not appreciably larger than that which can be measured directly. In general, the multiple-objects method described by Youden<sup>42,43</sup>, with mandatory use of Hart's tables<sup>44</sup> as recommended by Bennett,<sup>45,46</sup> should prove sufficient for the evaluation of most biochemical procedures.

Another conflict in requirements of the read-out system arises from the need for a low input impedance to ensure linearity while providing at the same time a short time constant to avoid distortions. The importance of this last point will become apparent after discussion of the dynamic performance of the complete apparatus. A solution of these problems, as well as those arising from the use of dissimilar transducers, requires the combination of several feedback methods.

A universal requirement is a low-input signal power drain. However, limitations on the current reduction are imposed by the matching input resistance of each transducer used. This resistance ranges from  $10^{-1}$  ohm (in thermocouples) to  $10^{12}$  ohms (in chemical electrodes). An acceptable range for barrier-type photocells is from  $10^2$  to  $10^4$  ohms. This can be increased to  $10^7$  ohms with photoresistive transducers and thermistors.

Despite such limitations, a chopped input AC amplification system was chosen. Preliminary experiments showed that the high impedance vibrating condenser modulation method, while satisfactory in potentiometric measurements, is not dependable for drift-free continuous recording.

To satisfy the modular design and to simplify maintenance, the input circuit was designed to consist of an amplifier\* that is commercially available and rather inexpensive. Any of the amplifiers described under this prototype is equally satisfactory. The few modifications required are chiefly external. The input transformer adaptation mentioned later can be made at the factory, and is required only when maximum available amplification must be obtained. The built-in stray-bucking circuit has little usefulness in the present application and must be readjusted carefully. The 12AT-4 input amplifier voltage tube, often the source of microphonic signals, is replaced by a premium tube 6201.† The 6X4 rectifier has always been satisfactory, but it can be replaced by a longer-lived 6X4-W. The second voltage amplifier tube, a 12AX-7, also must be replaced by a premium type 5751. Finally, the two 12AU-7 power tubes are removed and become available for use in the DC amplification stage that follows. In their place, a test plug is inserted into one of the sockets and a 4-wire shielded cable is brought to pins 9 and 4 (heater) and 1 and 6 (power), respectively, to supply the required voltages to the DC stage.

The output of the AC stage available at the test jack contains a 60 cps sine wave plus a small unmodulated component that must be compensated. The remaining sine wave is then rectified into a 120 cps ripple, an amplitude-modulated voltage whose phase no longer depends on that of the input signal. This output is suitable for further DC amplification.

The choice of amplification method is determined by the applications contemplated. In the first place, the correct reconstruction, both in shape and phase, of a given input wave is essentially a problem of time-integration rather than filtration. Refined techniques are available for this purpose. In the case of relatively low frequency signals, a single modulating chopper, as used here, is satisfactory if its frequency is much larger than that of the signal. It is demonstrated that, in such cases, the method of postdetection integration allows the measurement of modulated signals appreciably weaker than the initial background random noise, particularly if a partial DC feed-back loop is established at this stage (DC amplifier input). A plug-in detector unit satisfying these requirements incorporates a high-quality transformer (UTC-H-20) and two germanium diodes 1-N-92 in a full-wave bridge rectifier circuit. A relatively high current flows continuously into the secondary of the transformer and to ground, depending on the resistors connected. The output of this detector thus constitutes a virtual ground or point of fixed potential no longer affected by influences of frequencies and phases different from the demodulated signal. Such a connection has the advantages of a floating grid at very low signal level without its notorious instability, and it allows a much larger range of useful signals than does the floating grid.

The AC amplifier and its detector constitute a completely incoherent channel.

\* No. 40 X-356358, Brown Instrument Company.

† Radio Corporation of America, New York, N. Y.

A discussion of the band width of the system is interesting, but it is beyond the scope of this report. In such an amplifier, the output contains not only the steady component of amplitude  $2/\pi$ , but also the rectified components of all initially contained harmonics. Furthermore, the output signal:noise ratio is no longer equal to the ratio at the input (Smith<sup>47</sup>). The output noise voltage depends on the level of input signal, and the detection is necessarily nonlinear. Finally, the output noise band width is much larger than that of the detected signal, thus increasing filtration difficulties. The last difficulties are reduced by narrowing the input band width of the AC stage, as is customary, and by filtering its output at the detector. The wiring diagram of the amplifier and input transducer is given in FIGURE 10. The DC amplification stage shown includes a driver stage followed by a push-pull amplifier. Actually, the driver stage alone is perfectly satisfactory for measurement and recording. The addition of the push-pull stage merely adds to the total amplification without altering the operating principle. Therefore, the following discussion is concerned primarily with the driver stage performance. The DC amplifier incorporates a modification of the cascode circuit. Attree<sup>48</sup> showed that very high amplification may be realized if the anode of the first tube can be kept at a sufficiently high voltage. The first half of a 12 AU-7 tube rectifies the 250 v. AC output of the power transformer of the main AC amplifier. Regulation is obtained by a grid filter. The lowest point of this circuit is about  $-16$  v. below ground. The cathode is at  $+98$  v. DC. A portion of the bleeder resistor is common to the cathodes of the 2 tubes and performs a stabilizing function. The output is taken between the cathode of the driver tube (the lower half of the same 12 AU-7) and ground. Although the cathode potential is no longer exactly fixed, difficulty is avoided by providing some adjustment of the input grid floating point so that a self-biasing cathode resistor may still be used. Thus, either a low-resistance meter or a strip chart recording potentiometer may be used for measurements. Automatic gain control and zero shift also may be incorporated in the cathode of the driver tube, since the return is at a point very negative relative to ground. The entire DC stage, including the input detector bridge, is built inside a 2- by 3-inch Vector turret. The driver tube DC amplifier sensitivity is a function of its grid-to-cathode transconductance and of the output meter resistance. With a microammeter of 250 ohms internal resistance and  $0.5 \mu\text{a}/\text{division}$  sensitivity, the sensitivity at the plate is  $5 \times 10^{-9}$  amp/v.

The rejection factor is defined by the variation of output signal  $E_0$  produced by a given variation of the operative potential of the grid of the DC stage or driver tube. With a gain of about  $\times 1000$  realized in the cascode circuit, a grid input circuit of 0.020 sec. time constant, and a grid resistor of 20 megohms, the impedance of this stage is approximately 8020 megohms. Transient, limited variations of this very high impedance cannot reflect upon the output impedance of the preceding stage which thus is operating virtually in open circuit: optimum conditions for voltage amplification are realized. The high reverse resistance of the rectifier IN92 further contributes to this result: the total output impedance remains almost independent of the gain and of the feedback factor. The combination of circuits around resistor  $R_4$ , which





is used to adjust the grid operating potential, results in a rejection factor of nearly 1000:1 and, hence, extreme stability of the amplifier.

The total chopper noise imposes a limit upon the performance of the amplifier. This noise is the sum of thermal, electrostatic, electrochemical, and magnetic effects originating in the input modulator. Electrochemical noise is negligible in a well-designed chopper. Microphonics may amount to several volts in plastic-insulated input cables, but are virtually eliminated by using cotton-covered, enameled wires. Thermal noise elimination requires that only one spool of hook-up wire be used in the construction of a given apparatus. Magnetic noise originates in the contacts. It is usually lower than  $0.6 \mu\text{v}$  at 60 cps across a load impedance of 1 ohm or larger, and may be considered constant. It is eliminated by a zero-shift method. The main factor is the electrostatic noise due to capacitive coupling between the signal input circuit and the driving circuit, frequently found to be about  $8 \times 10^{-12} \text{ v./ohm}$  at the input resistor: it decreases with this resistance. The sum of all noise at 60 cps remains below  $0.6 \mu\text{v}$  with an 0.01 to 1 kilohm range of input resistors, and reaches  $45 \mu\text{v}$  at 1 megohm. Performance is further improved by twisting and shielding the modulator input leads, by sound-insulating and shock-proofing its socket, and by placing 2 suitable Faraday coils around its housing to neutralize the magnetic noise. Stray AC pickups from the coil are canceled by a 2- to 4-v neutralizing AC potential between the chopper housing and chassis, with the required leads in open air *outside* the chassis. Reducing the band width of the amplifier also decreases the chopper noise. With a band-pass filter centered at 60 cps, the amplified noise drops below  $0.1 \mu\text{v rms}$  in a 1-megohm resistor. This last method is most effective but its scope is limited, for peak-to-peak voltage and frequency become canonically related variables for very small band-width values, and the measurements lose their significance. Other aspects of chopper-generated noise due to nonlinearity of systems that may exhibit more than one stable operating domain at a constant excitation frequency have been discussed in the contemporary literature.<sup>49-51</sup>

Consideration of the amplifier input impedance is of paramount importance in data-reduction systems where a unique amplifier must be scanned rapidly across a plurality of input transducers of differing internal resistances. Although the system described is designed for only two kinds of transducers, the experience gained has been invaluable. Ideally, in order to maintain the stability of such factors as sensitivity and calibration over long periods of time, the amplifier input impedance must be higher than that of any of the transducers used; this requirement obviously is difficult to meet. An alternative solution makes use of the increased impedance resulting from the use of feedback, provided this remains compatible with the kind of transducer used. Often, it requires complete isolation of the amplifier output and feedback circuit by means of suitable transformers (as in the Offner Data Reducer). This technique can be applied with the amplifier described by connecting a suitable transformer (Palmer type T-67) at the input of the DC driver tube; feedback is then obtained through a bridge demodulator phased on the input chopper. The increase of effective input impedance  $R$  of an amplifier by negative feedback is generally expressed by the multiplication factor  $(1 + AB)$ , where  $B$  is the feedback ratio and  $A$  is the open-loop gain of the amplifier. The effective

input capacitance  $C$  is decreased by the same factor. The time constant  $RC$  and half-power band width  $\frac{1}{2}\pi RC$  are affected in the same proportion.

In practice, however, when a barrier-type photocell is used conjointly with a fixed linearization shunt resistor  $r_s$  of low value, the time constant becomes equal to the product of  $r_s$  by the effective input capacitance, or  $r_s C_g / (1 + AB)$ , where  $C_g$  is the original amplifier input capacitance in open-loop connection.

It must be kept in mind, however, that while negative feedback effectively reduces the time constant for a steady-state performance to a degree predictable by Ohm's law, it is nevertheless ineffective in preventing spurious transient responses, which remain unpredictable unless the input distributed capacitance is compensated dynamically. Further improvements in this direction could be obtained by the introduction of negative capacity feedback techniques, which were not considered in this project.

The amplifier time constant must be taken into consideration at very low input signal levels, even though DC measurements are to be made. Currently, the performance of a Weston photonic cell is as follows: a potential of 250 mv is produced, at infinite load resistance, the internal cell resistance dropping to about 7000 ohms. Thus a current of 48  $\mu$ a is produced in a 300-ohm load resistor, and the potential drop is only 14.4 mv. For the type 586-RR cell, such figures are attained with an illumination of 15 ft-c (15 lumens/sq. ft.). If now the illumination drops to  $\frac{1}{1000}$  of the above value in the zone of linear response of the cell the current becomes 48.0 m $\mu$ a. With a capacitance of the order of  $10^{-9}$  F, the transit time of the electrons within the cell is of the order of  $10^{-7}$  sec. Thus the signal is already at the level at which the time constant of the transducer becomes the predominant factor, unless that of the amplifier can be made smaller (by reducing the input capacitance). The alternative would be the use of counting techniques.

For electrometric measurements, a very high input impedance is required to keep the current drain below  $10^{-10}$  amp and, preferably, below  $10^{-12}$  amp. A satisfactory performance can be achieved by manipulating the load impedance  $Z_{sec}$  on the secondary winding of the AC input transformer. This load consists chiefly of the leakage resistor of the first tube grid (about 0.250 megohm). The DC resistance of the transformer secondary is negligible (2000 to 5000 ohms). The DC reflected input impedance  $Z_i$  is given by the usual relation  $Z_i = Z_{sec} X n^2$ , where  $n$  is the transformer turns ratio. TABLE 1 gives the characteristics of some commercially available transformers. The DC input impedance is of the order of 1000 ohms. The tuned impedance at 60 cps,  $R_e$ , is evidently very much larger than the nominal resistance of the transformer primary winding.

It is much more convenient to adjust  $Z_{sec}$  to a specified performance than to alter the turns ratio of a given transformer. The data given show that, for instance, transformer No. 355507-2 could be connected to an input shunt resistor of about 5000 ohms without greatly affecting the tuned input impedance of the circuit. Only the chopper noise would be increased, but it still would remain at a tolerable level. Practically, input signals in the millivolt range can be measured with a current drain of the order of  $10^{-12}$  ohm/mv, by taking advantage of this large reflected input impedance at 60 cps created by the input transformer. For this purpose, a 1000-megohm series resistor is placed

in the chemical electrode circuit to the chopper, to form a potential divider whose other arm includes the reflected input impedance. The high-voltage amplification provided by the complete amplifier (several millions) makes this method possible. Proper precautions must be taken against drifts and stray pickup when the DC resistance between contacts of the modulators is raised above the 1-kilohm range. In all these applications, the amplifier input works at a very low resistance above ground.

The output read-out device also influences the performance of the amplifier. In the most general case, the amplifier output contains both AC and DC voltages, for the random output amplified noise voltage always has a finite frequency. Read-out ambiguity is due to the fact that a microammeter's sensitivity to current is approximately proportional to the  $\frac{2}{5}$  power of its resistance, while its sensitivity to voltage is proportional to the  $\frac{3}{5}$  power of that quantity. Thus, for example, a fivefold increase in current results in a twenty-fivefold increase in deflection. Discrimination can be achieved with a more constant sensitivity in favor of the DC component of the steady signal provided the internal resistance of the meter is as high as is compatible

TABLE 1  
CHARACTERISTICS OF SOME COMMERCIALY AVAILABLE INPUT TRANSFORMERS

Brown Co. Part no.	Input $R$ (one side, DC)	$n$	$Z_{sec}$ (megohm)	$Z_i$ (ohms)	$R_{sec}$ (ohms)	Tuned $R_s$ (ohms)
355567-1	30	1:16	0.25	961	2500	370
356326	450	1:16	0.25	1000	5800	7000
355567-2	750	1:4	0.25	—	3400	30,000

with the remainder of the circuit—that is, voltage measurements are recommended, where possible.

Next, read-out damping must be considered. The purpose of damping is to give a better presentation of small amplified signals in the presence of unavoidable random noise; thus it requires a time-averaging operation.

Textbook discussions of critical damping of meters are valid as a starting point, provided one deals only with DC signals at a noise level not higher than  $\frac{1}{10}$  of the signal. With greater amplifier complexity and high amplification, such elementary considerations are no longer adequate. For instance, it is common to observe with a critically damped meter, large ripples that disappear at or near resonance conditions (underdamping). One fact to be considered is that no amplifier can be linear at all frequencies: particularly at zero frequency. The amount of damping desirable varies with frequency. The second factor is that the measured time constant of the meter is a composite of mechanical inertia *plus* electric inertia. The first component is a true coulombic force: above a critical frequency the force is independent of velocity (current) and frequency; below, it decreases more slowly than velocity. The second component is, however, proportional to velocity at all frequencies. It follows that no single linear network can be designed to compensate inertia at all frequencies, including zero frequency. The usual method of approximating mechanical inertia with combinations of  $R$ ,  $L$ , and  $C$  components under

cathode-ray oscilloscope control probably could be improved greatly by the use of nonlinear elements such as varistors or capistors. The deficiency of all damping methods unavoidably results in output distortions. If the input is a slow triangular wave, the sharpness of the peak depends on the harmonics whose amplitudes fall off as the square of the frequency. For instance, a wave of 1-cm. amplitude and 5-cps frequency has a 25-cps component that is 0.4 mm. in amplitude and is easily drowned in noise unless adequate damping over this frequency range can be assumed confidently. This promising field should be investigated seriously in view of the importance of solving the damping

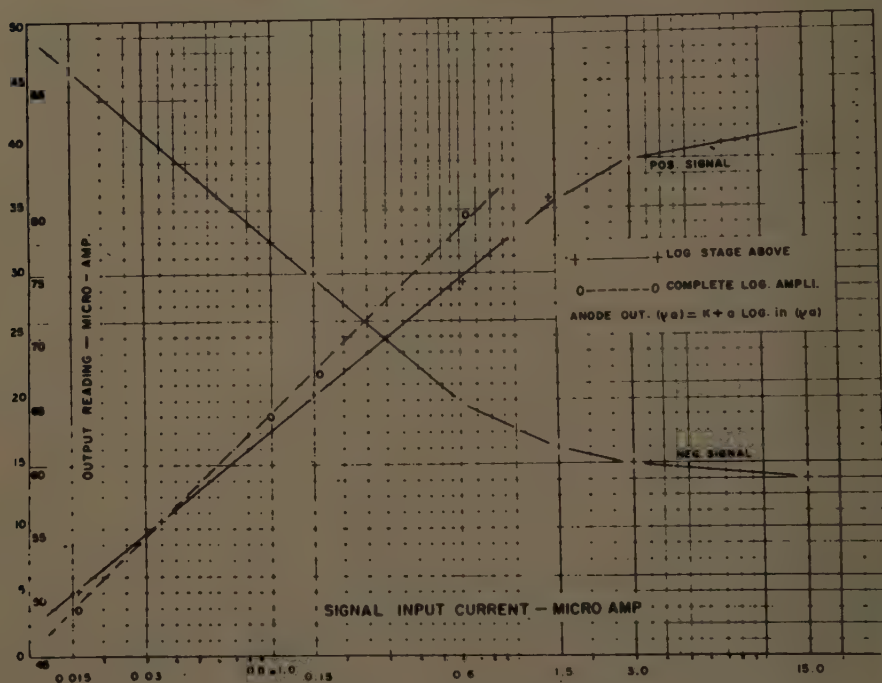


FIGURE 11. Amplifier linearity.

problem in applications involving automation equipment with a wide range of dynamic performance.

The linearity of the amplifier is illustrated in FIGURE 11, showing the relationship between output readings and the amplitude of test signals applied at the input.

The noise level shown in FIGURE 12 was recorded under the most unfavorable conditions (at 270 mμ with a 4-w Hg tube). It decreases very rapidly with the input shunt resistor value, and is negligible with 100 ohms. Such low resistance is used routinely for continuous colorimetry recording. Under these conditions the stability and long-term drift of the entire analytical system are very satisfactory, as shown in FIGURE 13, in which a similar record for a Beckman Model DU Spectrophotometer is compared.



*The Recorder*

Analogue data logging is currently performed by means of a conventional Brown strip chart-recording potentiometer of 2.5-mv. full-scale sensitivity and 2-sec. time constant. Its input load resistance is maintained at 500 ohms and it is center-grounded through a rectifier bridge to allow the use of the push-pull amplifier stage without further circuit alterations. Additional damping is provided by suitable condensers at the input terminals.

The only alteration is the addition of a double contacting arm on the hub of the balancing potentiometer. A pair of SPDT microswitches is mounted on a plate centered on the same hub and is made to follow the contacting arm.

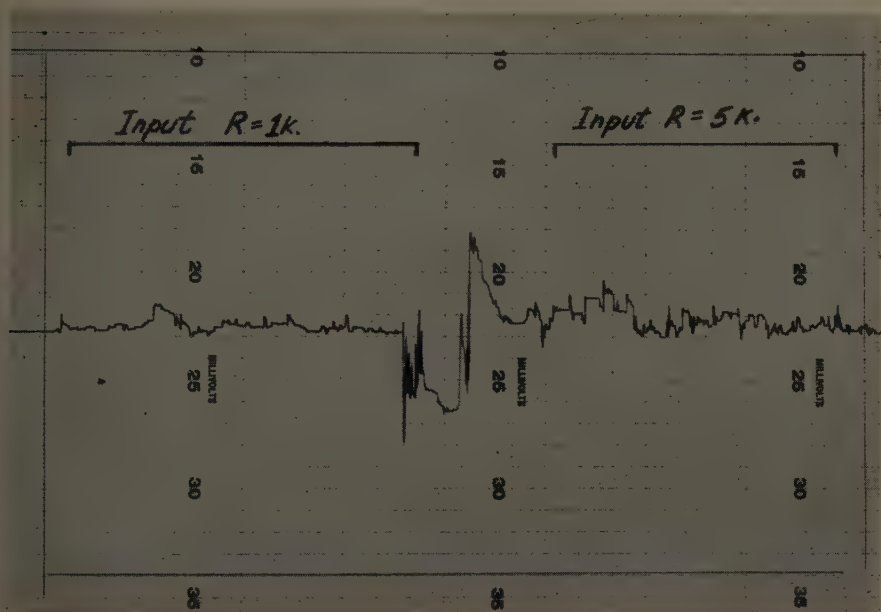


FIGURE 12. Amplifier internal noise versus input load resistance  $R$ .

Thus contacts are made or broken promptly every time the recorder stylus reverses its direction of travel. In this manner, it is possible for the instrument to record only peak deflections, omitting the irrelevant information contained in the rising and falling limbs of experimental curves. This feature allows the manual introduction of correction factors when required, as well as an easy serial digitalization of data related only to the calculation of chemical concentrations. This point will be taken up in a subsequent publication. FIGURE 14 shows the rear face of the recorder with the microswitches mechanism. A general view of the analyzer, including the fluid-processing and the measuring systems, is given in FIGURE 15.

*Dynamic Performance*

The economic necessity of speedy operation requires the application of a

suitable optimization method. It also places stringent specifications on the equipment.

The problems involved are conveniently approached by means of communication theory. The entire measuring system, extending from the measuring zone and primary transducer to the logger, is considered as an "incoherent linear noisy channel." The linearity of the amplifier response was discussed in the foregoing. The approximation to a linear system is not intellectually

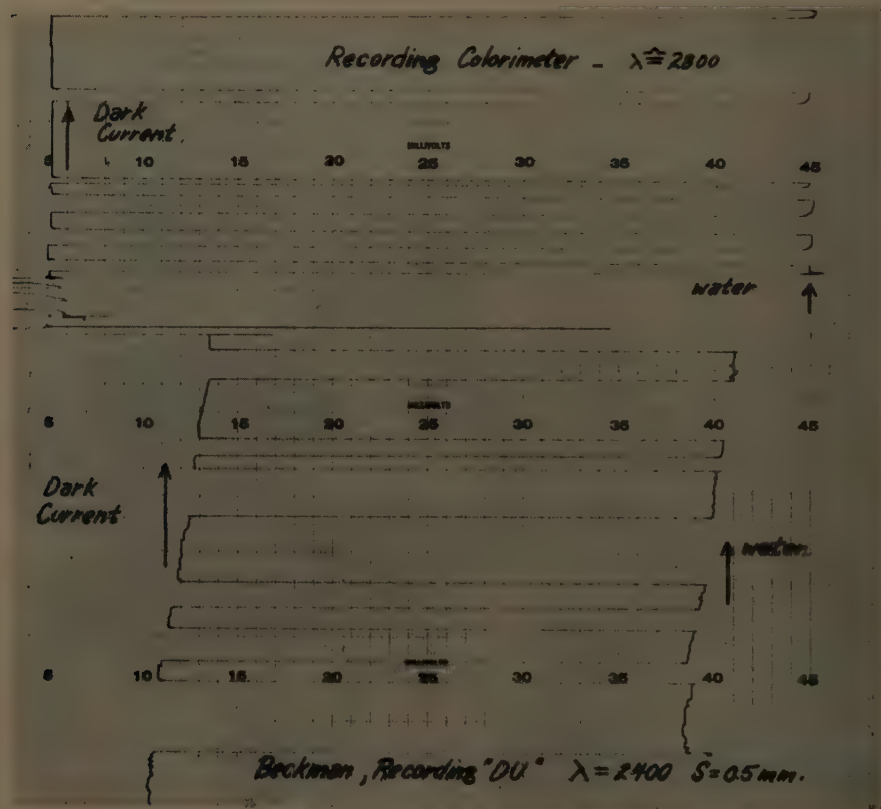


FIGURE 13. Amplifier stability. The recording colorimeter and a Beckman Model DU spectrophotometer are compared on the same Brown Instrument Co. recording potentiometer. Sensitivity: 2.5 mv full scale.

satisfying, but it is often sufficient in practice. A more complete theory should take into account the fact that no control system involving a feedback loop is linear. Trimmer<sup>52</sup> remarked that in such systems permitting power amplification at least one "property" of the system is made to vary, continuously or discontinuously, while the "quantity" is measured or controlled, the "quantity" being itself either a "variable" or a "property." It follows that the changing property of the amplification system can no longer enter as a simple coefficient in the differential equation describing the physical apparatus, but instead as a parameter dependent on one of the variables involved. The corollary is that "every feed-back system is (necessarily) non-linear." Such

views, unorthodox as they may be, lead to an interesting definition of the efficiency of a measuring system also proposed by the same author.<sup>53</sup> It may be that such evaluation methods will become mandatory with the expansion of the field of automatic analysis.

In the present case the problem is to determine the optimum speed of transfer, from the carrier stream, of the minimum information content essential to a solution of the chemical problem at hand. Cybernetic theory indicates that it is not necessary to have *all* relevant information to solve a given problem but that, instead, only a minimum *I* is required. Optimum measurement performance should be limited to obtaining this minimum. Any excess infor-

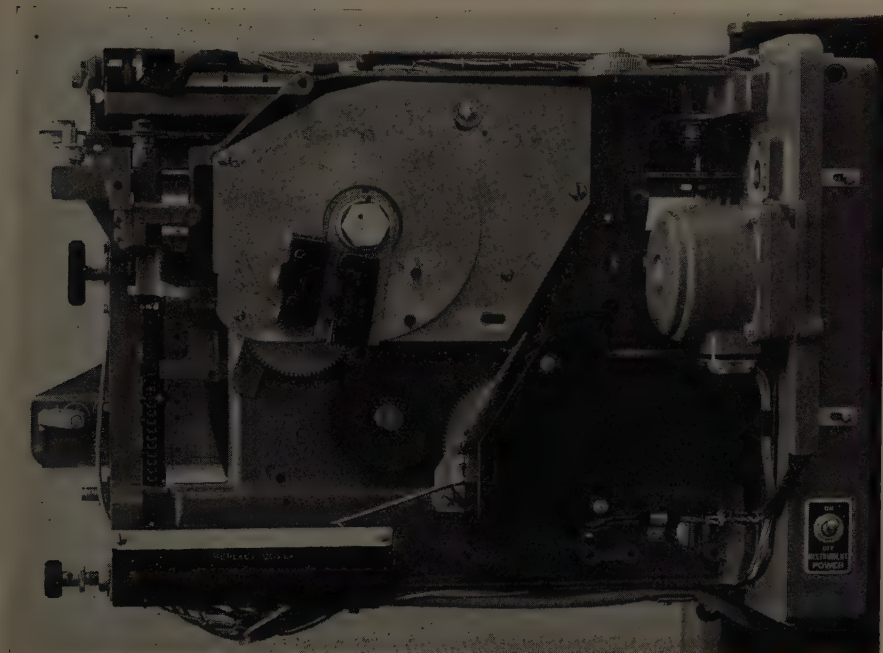


FIGURE 14. Recorder peak selector mechanism.

mation then contributes to the background "noise." This postulate is expressed by the probability-distribution functions of Wiener.<sup>54</sup> The a priori function of the probability density  $p_1(x)$ , with  $x$  either continuous or discrete,

$$I_1 = \int p_1(x) \log_2 p_1(x) dx$$

acquires a very sharp shape  $I_2$  in the a posteriori equation. The minimum of information obtained in the measurement is the difference between these two integrals,  $I_2 - I_1$ .

The communication theory treatment of this problem could be quite involved. However, a simplified approach leading to easy experimental verification was developed at length in a previous publication.<sup>55</sup> A linear system

of the kind considered is characterized fully by its low-pass cut-off frequency. This quantity is expressed in lines per unit length in the case of a spatial distribution. The treatment remains unchanged in the case of scanning operations whether the object or the scanning aperture is mobile, for only relative



FIGURE 15. Automatic analyzer, general view. Fluid processing and measuring systems.



motion is considered. It was then demonstrated that the cut-off frequency  $R_s$  is an instrumental characteristic that fully determines the first linear resolution limit,  $P$ , that is, the periodicity of the smallest spacing  $P$  that can be separated fully in the instrument's output. Thus,

$$R_s = 1/P \quad (4)$$

where  $P$  is expressed in units of length (periodic object spacing). This concept is akin to the distance function introduced in *Science* by Frechet in 1906 and detailed more recently by Zadeh.<sup>56</sup>

The quantity  $R_s$  is related to the numerical aperture of the instrument. In the case of colorimetric measurements on a continuous fluid stream of discontinuous composition, the aperture is defined by the geometry of the measuring zone or cuvette, considering only the length of the window  $A$ , in the direction of flow. It is found that the periodicity of the "moving object" thus scanned ceases to be detected by the instrument when the ratio  $A/P$  is numerically equal to the ratio of the first root of the first-order Bessel function (when  $J \times 3.83 = 0$ ) over  $\pi$ , or

$$A/P = 1.22 \quad (5)$$

This value corresponds to a sine-wave resolution  $W_0$  (in radians per unit length) given by:

$$W_0 = 2\pi/P = 3.83/A \quad (6)$$

Thus, the length of the measuring zone must not exceed the periodicity by more than a finite factor: as long as  $A \leq 1.22P$ , the latter quantity may be detected.

Next, the quantities  $A$  and  $P$  are related to the discontinuity of the carrier stream composition and to the over-all pumping velocity, since the scanning aperture (window) is stationary.

Several cases must be considered:

(1) The volume of solutions available is large, and one need not be concerned about the shape of the chemical zone front edge. However, the operating time may become uneconomical.

(2) The chemical zone to be analyzed includes a more-or-less sloping front followed by a large, indefinite column of homogenous material. Optimum operation results when successive chemical zones are just long enough to remain at, or slightly above, the resolution limit defined by EQUATION 5 and by the shape of the zone front edge.

(3) The chemical wave includes a sloping front, a negligible body, and a trailing edge possibly symmetrical with the front edge. The volume is insufficient to fill the entire measuring cuvette, but the height of the concentration wave is still proportional to the peak to be measured.

(4) The chemical wave is shaped as in the third case, but its total volume is small and the crest of the wave remains below the peak to be measured. Can the true concentration be evaluated with a finite degree of certainty, and under what conditions?

In the second case, the moving front edge determines the object's half-spacing or  $P/2$  (the spatial frequency being  $\nu = 1/P$ ). To resolve the output, it is sufficient that the repetitive frequency of presentation of such a chemical front along the window length  $A$  remains smaller than the time constant (or minimum measuring cycle) of the entire measuring system alone, the quantities  $P$  and  $A$  being related as shown in EQUATION 5. Thus  $P$  can now be evaluated quantitatively in terms of the pumping rate determining the frequency of presentation to the transducer.

Assuming that the chemical boundary remains relatively undistorted during transfer, the concentration gradient in the measuring zone may be as long as  $(A/2) \times 1.22$  over a full measuring cycle without appreciably affecting the final peak reading.

Obviously,  $A$  and  $P$  are related to the volume of the measuring zone by a constant factor, so that the over-all pumping rate has no influence upon their ratio.

To apply the dynamic method economically, no time should be lost between measurements. The same reasoning shows that the speed of presentation of successive samples, or the time interval between them, should be longer than the time required to pump a volume corresponding to  $(A/2) \times 1.22$  per measuring cycle, in order for the instrument fully to resolve the successive peaks. Experiment fully confirms that, in the case of colorimetric analysis, it is not necessary to return the measuring system to zero between successive measurements in order to record correct absorbancy peaks (see FIGURE 19), assuming that diffusion and chemical contamination between successive waves are negligible.

The last problem is the determination of the measuring system time constant or the minimum time required for a complete measuring cycle. For this purpose, the combination of the transducer, the amplifier, and the read-out device must be considered as one instrument whose merit is to be evaluated in each case. In the most general situation, a galvanometer or recorder with a rather long time constant is used. The time constant  $\tau$  is equal to the product of an electric resistance  $R_m$  multiplied by a capacitance  $C$ , provided  $R_m$  is very much smaller than the output impedance  $R_0$  of any electronic stage immediately preceding the meter or recorder. If this condition is satisfied,  $R_m$  and  $R_0$  can no longer be altered, but  $C$  can be manipulated within wide limits to suit the requirements. The output band width,  $df_0$ , is given by the general equation

$$df_0 = \frac{\pi}{2} \int_0^\infty \frac{d\omega}{1 + \omega^2 + \tau^2} \quad (7)$$

which simplifies to

$$df_0 = \frac{1}{4\tau} = \frac{1}{4R_mC} \quad (8)$$

At this point it is convenient to introduce the Sampling Theorem. One considers that the measuring system is being presented with a train of input signals or pulses whose representative function  $f(x)$  does not contain frequencies

higher than the upper value  $R_s$  of the pass band extending from  $f_0 = 0$  over the interval  $df_0$ . Then the Sampling Theorem indicates that the function is determined completely by taking a series of  $N$  samples spaced only as  $\frac{1}{2}R_s$ . The successive signals then are resolved fully. It follows from EQUATION 8 that the *minimum sampling time* of the system, or shortest permissible duration of one measuring cycle, is

$$t = 4\tau = 4R_m C \quad (9)$$

Consequently, one may write

$$\frac{1}{2}R_s = 4R_m C = t \quad (10)$$

In other words, the actual sampling time  $t_s$  is that which allows the samples to be presented at such a rate that their period  $P$  at the resolution limit  $R_s$  (EQUATION 4) satisfies the above relation, that is, the sampling period  $P$  equals  $8R_m C$ .

The optimum operating conditions of the entire system are now defined by two equations:

$$P = 8R_m C \quad \text{and} \quad P = A/1.22$$

relating the pumping speed (in terms of cuvette length per cycle) to the low-pass cut-off frequency  $R_s$  of the read-out device.

It is evident that the time constant  $T$  of the read-out device is the limiting factor in setting an economic speed of operation of the system.

It was found experimentally that, with  $T = 2$  (for a recording potentiometer with a 2-sec. full-scale operating time), the maximum rate at which specimens can be presented without creating some ambiguity is about one every 15.5 sec.

With most damped meters and automatic recorders the time constant varies considerably with the input signal level, owing to both the sensitivity threshold  $E_0$  and the acceleration factor (angular velocity)  $f$  in the rotating mechanism. In the case of a servo-controlled recorder, this acceleration factor  $f$  can be defined by

$$f = d\theta_0/dt \quad (11)$$

the symbol  $\theta$  representing the angular shaft rotation during the period  $dt$  in response to an amplified error signal of amplitude  $e$ . This quantity  $f$  is proportional to the product  $ke$ , to a first approximation. The factor  $k$  is itself a product of "constants," some of which include such quantities as the net gain of the amplifier, the actual value of  $e$  if the system is not linear, and the sensitivity of the system, which is  $d\theta/de$  in the case of a potentiometer. It follows that the over-all time constant  $f/k$  varies also with  $e$ .

Furthermore, the existence of the threshold  $E_0$  requires a correction term in the expression of  $\tau$  as a function of  $e$  and of the output signal voltage  $E_s$  (the total output voltage  $e$  being the sum of the signal voltage  $E_s$  plus the total noise voltage), as follows:

$$\tau dE_s/dt = k(e - E_0) \quad (12)$$

Combining EQUATIONS 11 and 12 we obtain:

$$\frac{d^2\theta}{dt^2} + \frac{1}{\tau} \frac{d\theta}{dt} + \frac{k\theta}{f\tau} = \frac{k\theta_1}{f\tau} \quad (13)$$

which shows that we are dealing with an oscillating system.

Optimum performance therefore is achieved when this system is critically damped, that is, when  $f = 4k\tau$ . It is remarkable that the factor  $k$ , containing the amplified error signal level  $e$ , is carried to the end of the analysis. Conditions deviating somewhat from the above relationship may lead to spurious resolution, hunting, or relaxation oscillations, as the case may be.

Conversely, if the sampling period  $P$  becomes equal to

$$\frac{1}{f} = \frac{1}{4k\tau} = \frac{1}{4kR_mC},$$

the entire system becomes unstable. This condition should be avoided even for the highest value of  $e$  (and of  $k$ ) encountered in practice.

Experimental verification of the applicability of the Sampling Theorem is necessary with certain chemical systems, for instance, those entailing boundary interactions, such as occur in analysis of enzymes. A convenient experimental approach is mentioned elsewhere (page 678).

## RESULTS

### *Chromate Test Solution*

The system was tested initially with one of the solutions recommended by the United States National Bureau of Standards.<sup>57</sup> Dry potassium dichromate was weighed accurately and a stock solution containing 400 mg./l. of salt was prepared. Subsequent dilutions were made with 0.001 *N* NaOH at a *pH* of 7.5, at which alkalinity only the chromate ion is present and the absorption peak wave length does not vary with dilution. All measurements were made at 22° with a narrow band-pass glass filter peaking at 415 mμ. The concentrations ranged from 0 to 400 mg./l., the latter corresponding to a maximum optical density of about 1.0.

The performance of the proportioning system was tested simultaneously. Each dilution of the chromate solution was analyzed and then diluted further, in constant proportion with the NaOH diluent by the pumping system.

A complete experiment is illustrated in the unretouched record of FIGURE 16. The peaks represent per cent transmittancy relative to water. The uniform dilutions effected by the proportioning system are indicated by the X marks on the record. The figures to the right of these marks are the manually set dilutions. Each automatic dilution is framed by two reference points obtained with the same initial solution. The spikes on the tracing indicate the actual starting time of the automatic dilution operation and have no bearing upon the calculations reported. The chart speed was 20 cm./hr. Both ascending and descending concentration runs are reproduced for the sake of completeness.

The record demonstrates the excellent reproducibility of the measurements over the entire scale. The peak values were converted to absorbancy values,



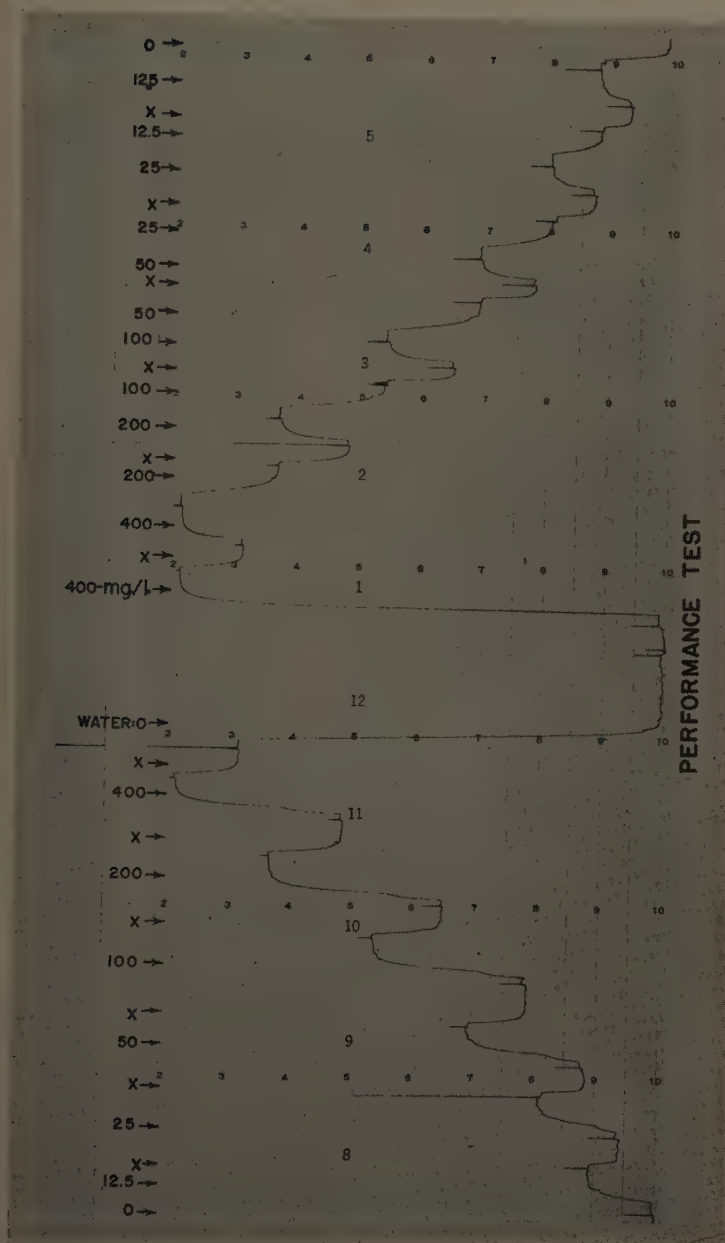


FIGURE 16. Automatic recording, Colorimeter performance test, with standard chromate solution absorbance relative to water versus mixing ratios, measured at 415 mμ.

and for each concentration an apparent proportioning ratio—the ratio of optical densities before and after automatic dilution—was calculated (*see* TABLE 2).

The accuracy of the proportioning system is satisfactory. The apparent proportioning ratio is constant up to about 40 mg./l., a range of concentration within which the solution obeys Beer's law. The small systematic deviation at higher concentrations is, in reality, a measure of the deviation from the logarithmic law. Such deviations are expected when a filter colorimetric technique is used, for the chromate solution absorbance varies very rapidly in the 415  $m\mu$  region. A change of 0.05 in the ratio may result from an error of 0.5 per cent in the absorbance. Under such conditions, the departure from

TABLE 2  
PERFORMANCE TEST WITH STANDARD CHROMATE SOLUTION

Dichromate concentration* (mg./l.)	Absorbancy	Apparent proportioning ratio
I 12.5	0.0495	
	0.0270	1.75
25.0	0.094	
	0.054	1.74
50.0	0.160	
	0.104	1.54
100.0	0.273	
	0.187	1.45
200.0	0.438	
	0.319	1.33
400.0	0.670	
	0.502	1.33
II 12.5	0.056	
	0.032	1.75
25.0	0.094	
	0.059	1.65
50.0	0.164	
	0.106	1.54
100.0	0.274	
	0.187	1.46
200.0	0.438	
	0.319	1.33
400.0	0.672	
	0.503	1.33

\* In terms of weighed-out dichromate salt.

linearity of the 400-mg./l. solution compared with the 12.5-mg./l. dilution ( $1.75 - 1.33 = 0.42$ ) represents a deviation of only 4.2 per cent in terms of absorbancy. Such an error is even less than was expected.

#### *Turbidimetric Analysis of Proteins*

Quantitative analysis of proteins by turbidimetry as currently carried out in the clinic is appealing on account of its technical simplicity. The procedure merely involves mixing the unknown specimen with a suitable reagent such as 3 per cent aqueous sulfosalicylic acid, the modified reagent of Exton (which contains, in addition to sulfosalicylic acid, 20 per cent sodium sulfate and some bromphenol blue indicator), or some other suitable sulfonic acid such as  $\beta$ -naphthalene. The resulting turbidity is observed after a specified time. Simi-

lar methods recently have been used for the fractional analysis of blood plasma and serum analysis in ammonium sulfate, sodium sulfate, or thiosulfate solutions of controlled  $pH$ . Despite their empiricism, such methods are generally in fair agreement with the results of electrophoretic separation and are quite satisfactory for clinical applications.

It is realized that turbidimetric measurements are affected by particle size and rate of growth which, in turn, depend upon many factors that can be controlled only with difficulty. Furthermore, measurement of opalescence is not as straightforward as measurement of color transmittancy obeying Beer's law. Nevertheless, it is possible to adjust the physical conditions so that measurements made with an ordinary colorimeter can be related to actual concentrations in a simple manner over a sufficient range of physiological concentrations.

Timasheff and Kronman<sup>58</sup> have shown that the degree of association of proteins, which greatly affects their biological and chemical properties, in general decreases rapidly with dilution below about 1.5 gm./l. The net result is a very rapid decrease in the light scattered at the higher dilutions. While this effect varies greatly with the nature of the system considered (typical systems are associated protein in water, isoionic protein in ion-free solvent, charged protein in ion-free solvent, and a multicomponent system containing other reactants), in every case the general equation contains a term  $H$  that varies inversely as the fourth power of the wave length. The scattered light also decreases in the same manner as a function of wave length. Under the conditions required for precipitation at the isoelectric point—low charge, high ionic strength, and little or no binding of the buffer components—most proteins behave as pseudo-two-component systems for which the mathematical approach is rather straightforward and the approximation just mentioned is satisfactory.

In addition, the screening effect, making the Debye-Hückel parameter  $k^2$  proportional to the square of the protein concentration, as shown with blood albumin by Doty and Steiner,<sup>59</sup> further decreases the amount of light scattered at higher concentrations. As a result of these factors, the contributions of diffusion and scattering to turbidimetric measurements at higher wave lengths become negligible, even in dilute solutions.

The determination of albumin in urine by this procedure requires a calibration curve made with pooled urines, establishing the average natural color of this fluid. High precision would require subtracting an individual blank of the same specimen diluted with water. Some spectrophotometric determinations of urine color versus wave length were performed with a Beckman Model DU Spectrophotometer and automatic spectral energy recording attachment. The average color of urine at one-third dilution represents an absorbance that is 0.040 at 640  $m\mu$  and decreases rapidly at longer wave lengths, vanishing at 750  $m\mu$ . Replacing the water by 5 per cent sulfosalicylic acid solution has a negligible effect upon the color. In terms of albumin concentration, the above value of absorbance represents a fictitious value of 7 to 9 mg. per cent, taken into consideration by the average pool used for calibrations. Only approximately 10 per cent of all urine specimens do produce a higher blank value, of which half (5.0 per cent) thus produce an error of only 3 mg. of fictitious albumin per 100 ml. An occasional very dark specimen may

produce, after dilution, a blank value as high as 0.070 at 640  $m\mu$ , representing about 10 mg. per cent of albumin, an excess of 3 to 5 mg. over the corrected average. Such a value would require compensation by means of an individual blank measurement. Such small incident and systematic errors are usually insignificant except in borderline insurance cases where the constancy of the findings must be verified.

The experiments detailed herein were performed with a filter peaking at 640  $m\mu$ , although more recently another filter with a maximum at 680  $m\mu$  has become available.

The automatic measurement of urine albumin by turbidimetry illustrates well the almost universally required adaptation of the procedure to the equipment for optimum performance in preference to exact simulation of the chemist's performance with test tubes. To ensure reproducible results, it is necessary to consider the following factors: reagent concentration, proportioning ratio, albumin concentration, fluid transit time, which determines the size of dispersed particles admitted to the measuring cuvette, over-all pumping rate, and minimum sample volume.

In the following experiments, standardized protein solutions were prepared by diluting with physiological saline solution pools of fresh human sera analyzed initially by the biuret method. It was found that such dilute protein solutions were stable for less than thirty minutes, when the measurements were no longer reproducible. Such findings are similar to my previous observations<sup>60-64</sup> relative to the instability of the refractive index of blood proteins in dilute solution due, presumably, to a change in the state of association of the globulin fraction.

A detailed investigation of the effects of the factors listed would be time-consuming. Fortunately, it is possible to devise a preliminary operating procedure of such character that the automatic analyzer traces a family of curves from which the conditions required for optimum performance can readily be specified. Optimizing the performance thus requires compromising between maximum sensitivity, reproducibility, speed, and minimum sampling volume. A generally applicable method is developed below.

First, the optimum sample volume of a protein solution is determined with an arbitrary concentration of reagent and an arbitrary mixing ratio. The experiment is performed with at least two protein concentrations that lie close to the expected range limits of the method. The records illustrated in FIGURE 17 show that the optimum volume for dynamic operation yields a transmittance 50 to 100 per cent of the value obtained under complete equilibrium conditions.

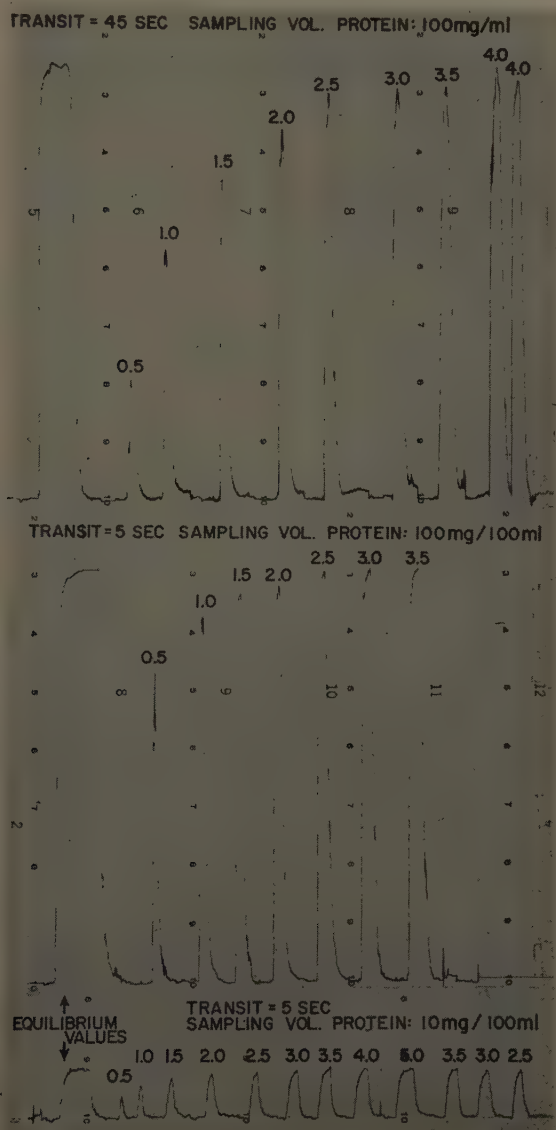
Reversing the mixing ratio without altering the concentrations (not illustrated) resulted in a rather steep curve and a loss of sensitivity at the lower sampling volumes. Thus the conditions illustrated are those for which the sampling error is minimized at the cost of a slight loss in sensitivity. Similarly, the sensitivity could be regained by increasing the transit time from 5 to 45 sec., but the sampling error would again become larger.

Next, the optimum concentration of reagent is evaluated by running continuously a solution of protein whose concentration is near the maximum expected and injecting various concentrations of the reagent solution while maintaining



the transit time and mixing ratio, previously determined, at constant values. A record of such an experiment is illustrated in FIGURE 18. The optimum concentration lies between 4 and 5 per cent sulfosalicylic acid, further increases resulting in negligible gain.

The conditions ultimately adopted are as follows: a sample/reagent mixing



ratio of 3/5, a reagent concentration of 5 per cent, a transit time of 5 sec., a time lapse of 15 sec. between successive determinations, and a measurement wave length of 640  $m\mu$ .

FIGURE 19 illustrates a series of experiments performed under nearly optimum conditions. Experiment I is a tentative calibration curve of per cent transmittance versus albumin concentration at equilibrium, measured at the end of 1 min. The samples were presented to the machine in order of increasing con-

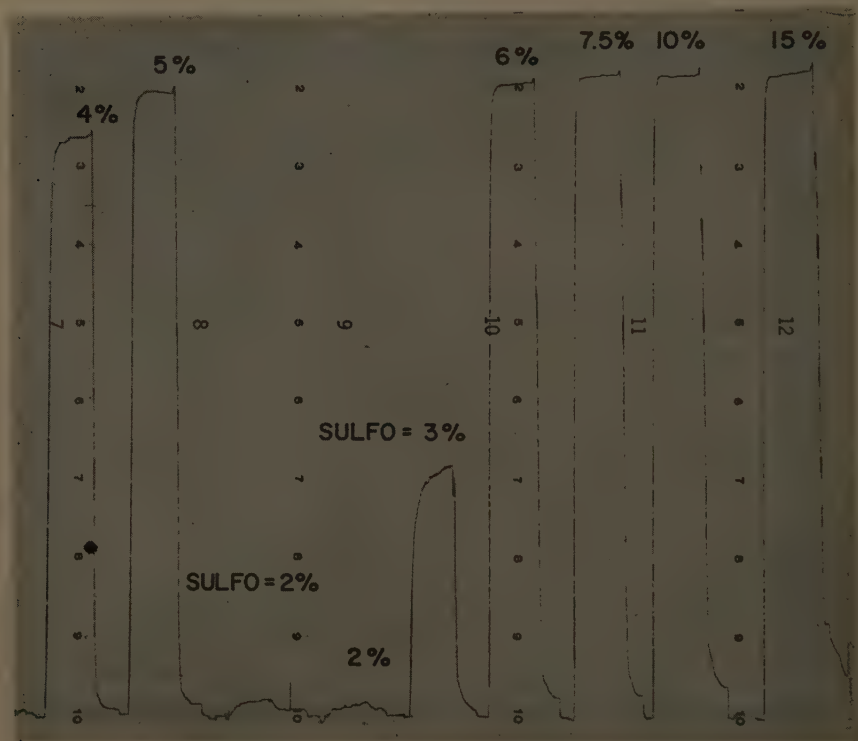


FIGURE 18. Automatic albumin analysis. Determination of optimum reagent concentration for a sampling ratio of 3/5, an albumin concentration of 100 mg. per cent, and a transit time of 45 sec.

centration, and the cell was flushed thoroughly between measurements. Experiment II is similar to Experiment I, except that the samples were presented randomly. Experiment III was conducted in the same manner, except that the flushing step between measurements was omitted. In these three experiments, the sampling volume was 3.0 ml. and the total transit time was 45 sec. A comparison of Experiments I and II reveals the invariance of results with the order or presentation of the samples of different concentrations. A comparison of Experiments I or II and III shows that it is unnecessary to clean the measuring cell completely or to return the instrument to zero between measurements. Thus the successive plateaus on tracing III represent a complete,

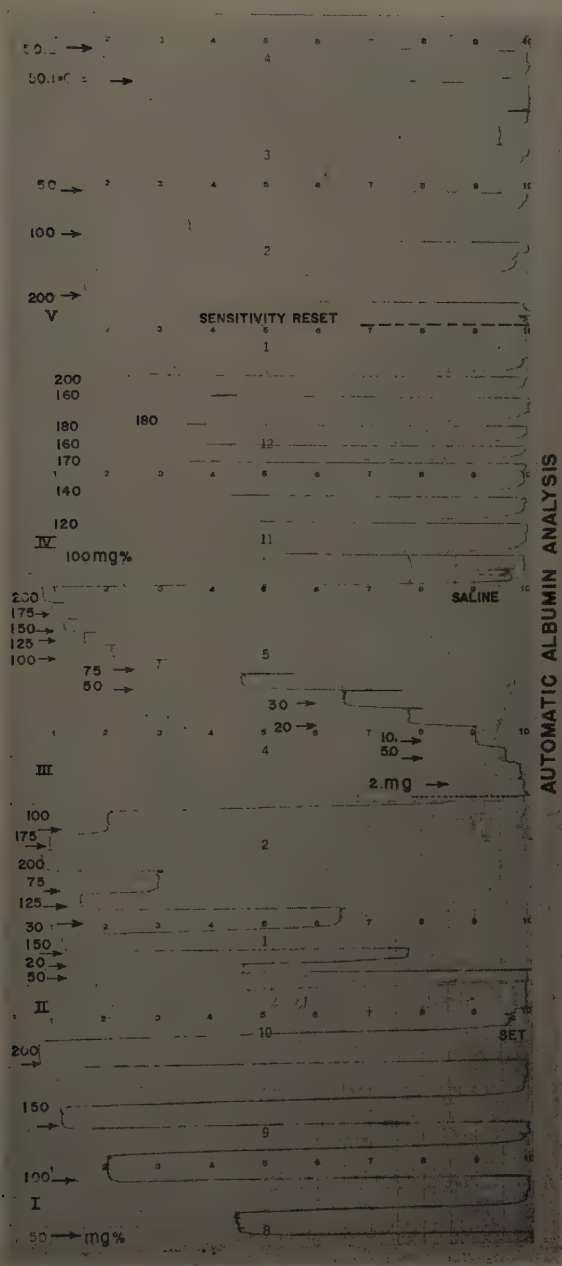


FIGURE 19. Automatic albumin analysis. Comparison of static and dynamic calibration methods.

*equilibrium* calibration curve extending from 0 to 200 mg. albumin per 100 ml. At the pumping rate used, equilibrium was reached with a sampling volume of 3.0 ml. Note the increased sensitivity (scale expansion) at the lower, clinically important concentration range from 0 to 50 mg. per 100 ml. In this range a concentration of 2 mg. per cent is discernible over the background noise. Experiment V shows a few check points obtained under the conditions of Experiment II. The time spanned by experiments I through V was more than 7 hours. Thus the long-term stability of the analytical system was demonstrated.

For comparison, Experiment IV was conducted with a transit time reduced to 5 sec. and a sampling volume of 0.5 ml. In such experiments, the per cent transmittance characteristic of each specimen is caught in transit rather than at equilibrium. Such *dynamic* calibration curves are remarkably reproducible. Although a loss of sensitivity results, the analysis time is reduced by a factor of 4 and the other factors are unaffected. The loss of sensitivity can be evaluated by comparing the peak optical absorbance measured at equilibrium with

TABLE 3  
URINE ALBUMIN ANALYSIS: INFLUENCE OF SAMPLE VOLUME ON SENSITIVITY

Albumin (mg./100 ml.)	Sample (ml.)	Absorbancy	Ratio
10	3.0	0.0250	1.72
	0.5	0.0145	
50	3.0	0.215	1.60
	0.5	0.134	
100	3.0	0.450	1.67
	0.5	0.268	
200	3.0	0.810	1.39
	0.5	0.580	

a 3.0-ml. sample to that resulting from the use of a 0.5-ml. sample under dynamic conditions. Such a comparison is given in TABLE 3.

The loss of sensitivity with smaller volumes is rather constant, except at the 200-mg. concentration, for which the loss is considerably less. It is evident from these data that the accurate analysis of albumin by turbidimetry becomes possible with a sample volume as small as 0.5 ml., and that recorded results are available within a total processing time of little more than 15 sec.

#### *Urinary Glucose Analysis*

The following procedure is offered as an example of the rather complicated problems that could be solved by the automatic analyzer described.

Clinical methods of sugar determination, with the exception of enzymatic tests, establish at best statistical associations between a certain kind of reducing power and the probable occurrence of glucose at a specified concentration in an individual presenting other suspicious clinical symptoms. With these methods, glucose is not titrated with any degree of specificity. Consequently, it is conceivable that the degree of association could become loose if nonglucose-reducing substances unexpectedly were present. That this is too often the case is apparent when a sufficient number of specimens is analyzed simultane-



ously by several methods. Such an investigation was conducted in this laboratory on 4536 random preserved urine specimens. The detailed results will be reported elsewhere. Of this total, 22 specimens were found to exhibit no reducing power when tested with dinitrosalicylic acid, while the oxidase method revealed reductions of more than 100 mg./ml. Similarly, 76 specimens were found to have a total reducing power between 200 and 700 mg. per cent, while their oxidase reactions were completely negative and no glucose could be identified, even after chromatographic separation. Finally, 138 specimens showed a total reducing power of 200 mg. per cent or more in a negative enzyme test, while glucose could be identified at the 50- to 100-mg. level by either chromatography or fermentation.

These results illustrate the nonspecificity of the clinical method routinely employed, as well as the interference between the specific oxidase method and the nonsugar-reducing substances. The latter may be excreted in some cases in large amounts entirely unrelated to the presence of true glucose. Such findings necessitate revision of aphorisms commonly found in the biochemical literature such as "glucose is frequently present when a high reducing power is detected." Similarly, the relationships between fasting glucosuria and normal glycemia in diabetes, and the delayed dip below the fasting value in certain types of glucose tolerance curves, all determined thus far by nonspecific methods, need to be re-evaluated.

With the paucity of available information, it is convenient to discard the nonglucose-reducers as being physiologically unimportant, as is done in many textbooks. However, if one assumes that nothing in nature is likely to be unimportant, it becomes interesting to evaluate quantitatively the relationships between these two types of reducing agents. In the method developed for this purpose, the nonglucose reducers are oxidized in 0.1. ml. of urine by 0.2 ml. of a saturated solution of K biiodate in 0.55 ml. of K phthalate buffer ( $pH = 5.0$ ) at  $70^{\circ}C$ . for 2 min. Iodine is liberated in this reaction. After cooling, the iodine is complexed with 0.1 ml. of a 20 per cent ammonium acetate solution. The reaction takes 1 min. Glucose, if present, is unaffected by this treatment. It can be titrated by the addition of 3.0 ml. of a reagent consisting of 0.01 per cent peroxidase, 0.125 per cent aerodehydrogenase, 5 per cent ammonium sulfamate, and 0.01 per cent *o*-tolidine in 0.05 *M* K phthalate buffer at a  $pH$  of 5.0. A blue color develops in 1 to 4 min., depending on the glucose concentration. The color is measured at 640  $m\mu$ .

The same oxidation reaction can be applied to determine the urinary nonglucose reducers. Indeed, the iodine liberated is proportional to the reducing power, and is used to develop a blue color directly. The reagent used is that described above with ammonium acetate and the enzymes omitted. The successive steps involved are illustrated in the form of a block diagram in FIGURE 20. The same diagram shows the fluid pathways for the two reactions. Ammonium acetate was chosen for complexing with iodine from thirty compounds effective at various concentrations. This agent acts at a relatively low concentration and in a short time. It is devoid of inhibitory effect upon the enzymes, and it has little influence upon the rate of oxidation of the chromogene. Other incidental studies dealt with the effect of such factors as buffer

composition, strength and  $pH$ , oxygenation, temperature, nature, and concentration of chromogene, role of anions, role of impurities, and influence of urine preservatives. The addition of ammonium sulfamate greatly enhances the sensitivity of the *o*-tolidine base reagent in the presence of peroxides, but is unnecessary when the highly purified sulfate is available.

The amount of color developed at any moment is the algebraic result of simultaneous color-intensification and color-quenching reactions that are affected in the same direction but at different rates by the glucose concentration. This phenomenon is illustrated in the unretouched records of FIGURE 21. Such records of color intensity versus time permit determination of the optimum

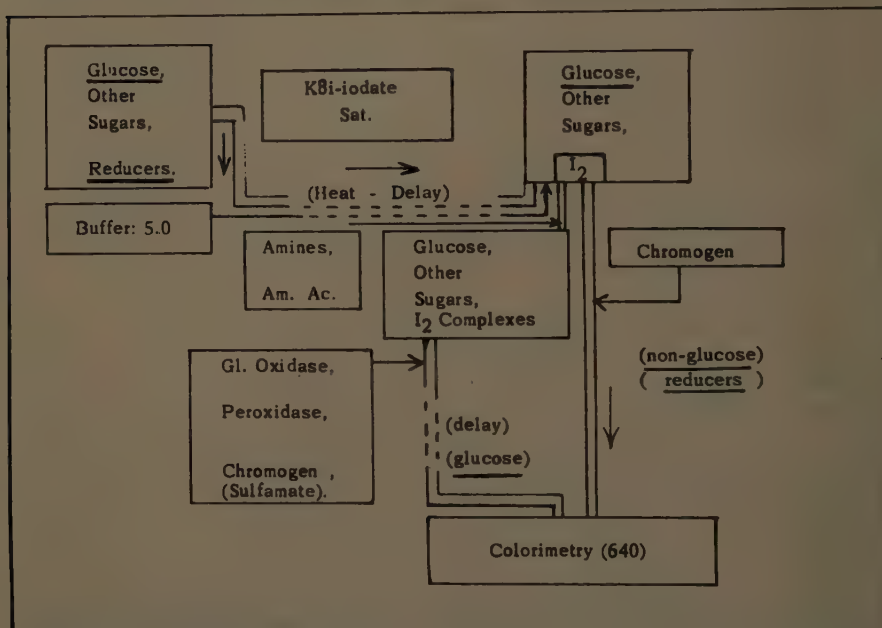


FIGURE 20. Automatic urine glucose analysis. Modified aerodehydrogenase method. Block diagram and flow sheet.

reaction time at the end of which the error in absorbancy due to small timing uncertainties is reduced. The best reaction time is between 2.5 and 3.0 min. after the addition of the enzymes at room temperature. Under these conditions, the plot of absorbancy versus glucose concentration obeys Beer's law from 0.0 to over 0.750 gm./100 ml., as shown in FIGURE 22

The plot shown is in reality a composite of six independent experiments with pooled urine specimens previously analyzed by the Somogyi-Nelson method, to which glucose in known amounts was added to aggregate the figures shown on the diagram. It is to be noted that the deviation from Beer's law at low sugar concentrations reported by other authors was not observed in these experiments.

#### *Red Blood Cell Count*

A number of physical methods have been offered as substitutes for the

tedious visual counting of red blood cells (RBC) in the clinical hemacytometer. These procedures include T. Young's eriometer (ca. 1820), the Hayden Hausser erythrocytometer,<sup>65</sup> the diffractometer of Cox and Ponder,<sup>66</sup> and others. They have been discussed at length in the medical literature.<sup>67</sup>

The method of Blum<sup>68,69</sup> is used widely when a photoelectric colorimeter is available. The colorimetric method may be considered satisfactory, although

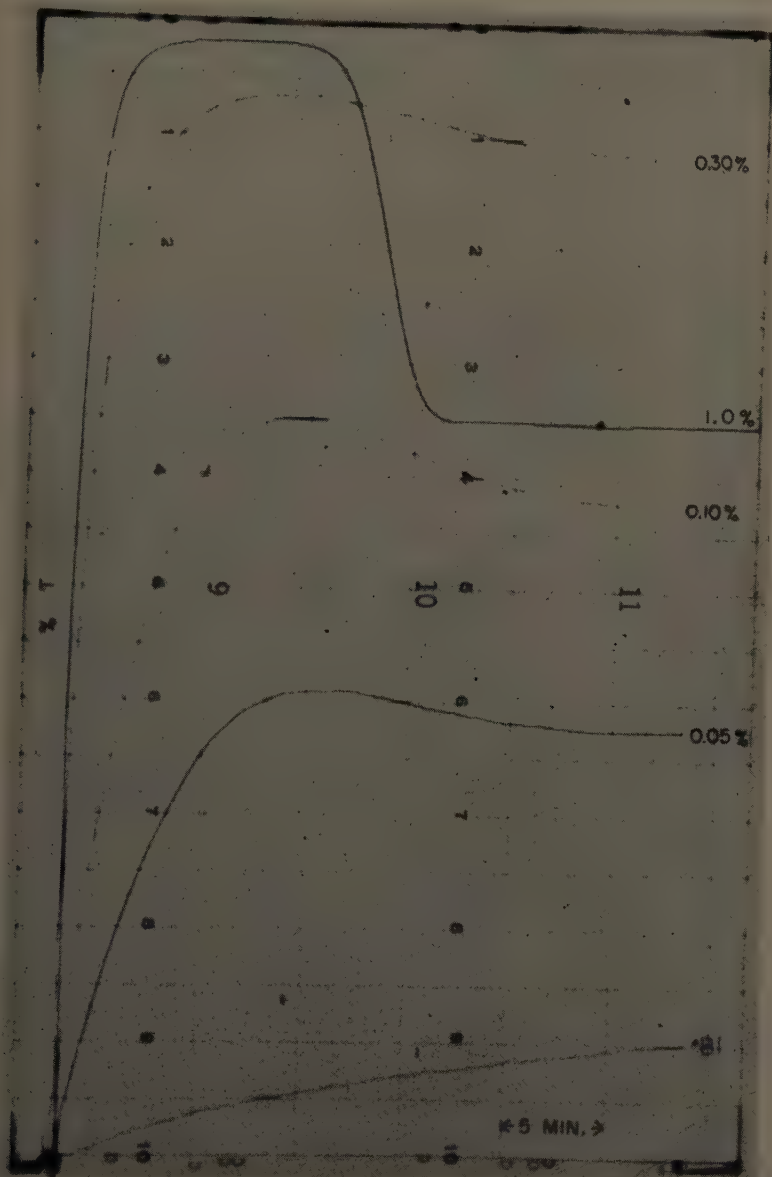


FIGURE 21. Automatic urine glucose analysis. *o*-Tolidine blue color development versus sugar concentration.

for screening purposes only, when anisocytosis is absent, when the mean corpuscular volume is close to normal, and when the color index lies between 0.8 and 1.1. These limitations might be eased by the use of "sphering agents" which, however, have been available for such a comparatively short time that clinical experience in their use is still lacking. Compared to these sources of error, it makes little difference whether saline solution, Hayem's fluid, or Gower solution is used for blood dilution in these measurements.

Oxalated normal blood diluted with saline was used throughout the following experiments. Initially, the RBC count was determined with the hemacytometer. The lowest dilution made was 2:500 instead of the customary 1:500.

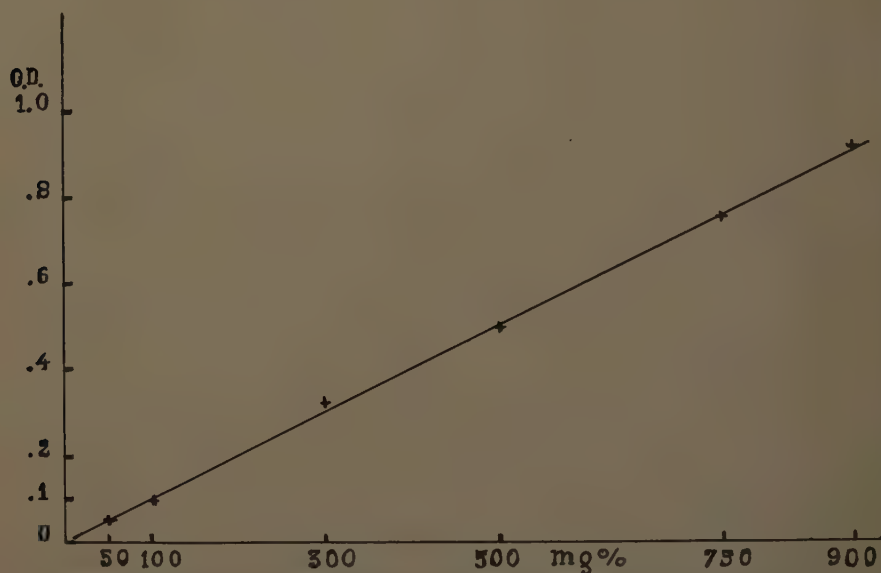


FIGURE 22. Automatic urine glucose. Absorbancy versus glucose concentration (recovery experiment).

The diluted suspensions then were pumped at a uniform rate into a continuous stream of diluent (saline) at a proportioning ratio of 0.6:1.0, so that the measuring cuvette received an RBC suspension comparable with that currently employed in routine clinical determinations. The peak transmittances were measured at  $520\text{ m}\mu$  against a saline blank, and the chart readings subsequently were converted to the corresponding absorbancy values  $A_s$ . TABLE 4 offers a comparison of the values of  $A_s$  versus RBC count obtained with a Coleman Jr. colorimeter, a clinical Weston-Rouy instrument, and the automatic analyzer described.

A round measuring cuvette of 12-mm. equivalent diameter is used with the Coleman instrument, yet the sensitivity is considerably lower than that of the other instruments used. It is to be noted that measurements with the Weston-Rouy Colorimeter were made at  $490\text{ m}\mu$ , with a corresponding increase in sensitivity. In each case, the representative diagram is a fairly straight line.



The accuracy of the instrumentally made dilutions was tested further. Two series of blood dilutions were prepared as before, with initial values of 2:500 and 4:500, respectively, or a ratio of 1:2 between the two series. Measurements were made as previously described. TABLE 5 gives the detailed results. An error of 1 per cent on  $T_e$  at 50 per cent transmittance results in an uncertainty of  $-0.054$  to  $+0.068$  in the ratios reported. Consequently, the absorbance ratios can be considered sufficiently constant within the limits of

TABLE 4  
RED BLOOD CELL COUNT BY COLORIMETRY: COMPARATIVE SENSITIVITIES OF  
THREE MEASURING INSTRUMENTS

RBC count (10 <sup>6</sup> /mm. <sup>3</sup> )	Absorbancy		RBC (10 <sup>6</sup> /mm. <sup>3</sup> )	Absorbancy, automatic analyzer
	Coleman Jr.	Weston-Rouy		
0.375	—	0.038	0.3906	0.051
0.750	—	0.076	0.7812	0.096
1.500	0.100	0.153	1.5625	0.187
1.800	0.119	0.184	1.8624	0.215
2.400	0.155	0.244	2.4640	0.292
3.000	0.194	0.301	3.1248	0.368
3.580	0.229	0.357	3.7248	0.438
4.240	0.273	0.413	4.2848	0.515
4.540	0.292	0.444		
5.140	0.328	0.495		

TABLE 5  
RED BLOOD CELL COUNT BY COLORIMETRY: DEVIATION FROM LINEARITY  
VERSUS NUMBER OF CELLS COUNTED

Series a RBC count (10 <sup>6</sup> /mm. <sup>3</sup> )	Absorbance		Absorbance ratio b/a
	Series a	Series b	
0.3906	0.0505	0.096	1.90
0.7812	0.094	0.185	1.96
1.5625	0.174	0.315	1.81
1.8624	0.204	0.383	1.87
2.4640	0.257	0.456	1.77

measurement accuracy. The discrepancy between the average of these ratios, 1.862, and the expected value of 2.0 probably represents a compendium of initial blood pipetting errors plus the effect of small deviations from an exact logarithmic law at the higher blood concentrations.

Still another approach to RBC counting was attempted with this automatic analyzer. Uniform aliquots of the blood dilutions used in the previous experiment (TABLE 4), representing one tenth of the volume originally available, were pumped through the apparatus at a uniform speed at the proportioning ratio used previously (0.6:1.0). Thus various volumes of RBC suspensions were used, all containing the same absolute number of cells. The measurements were effected as described previously, and the recorded curves were cut

out. The curve areas, corrected for the blank value, were evaluated by determining the weight of the cut-outs on the analytical balance. Thus figures were obtained that were expected to be proportional to the total number of cells contained in the volumes of fluid used for measurements. The results are given in TABLE 6.

Obviously, sample volumes less than 1.50 ml. are insufficient to fulfill the condition of EQUATION 5. Nevertheless, the ratios of curve areas, averaging 1.656, are remarkably constant for all volumes used. These results indicate that the areas under the recorded curves are, after calibration, proportional to the RBC count within a limited range, provided the sample volume is held constant. The variation of area with dilution at constant RBC count is relatively small in both series of measurements. It probably represents the effect of the masking factor, which is relatively more important at higher dilutions at which, simultaneously, experimental errors become larger. The discrepancy between the recorded area ratio of 1.65 and the expected value of 2.0 is probably

TABLE 6  
RED BLOOD CELL COUNT BY COLORIMETRY: CURVE AREA VERSUS  
CELL NUMBER RELATIONSHIPS

Volume (ml.)	Series <i>a</i> RBC count (10 <sup>6</sup> /mm. <sup>3</sup> )	Weight of area		Weight ratio <i>b/a</i>
		Series <i>a</i>	Series <i>b</i>	
0.5	1.5625	52.9	88.7	1.676
0.75	1.1718	58.9	96.4	1.639
1.00	0.7812	59.6	98.8	1.658
2.00	0.3906	64.0	106.0	1.656
4.00	0.1953	70.0	115.5	1.650

related to diffraction phenomena in the anisotropic suspension and to some factor depending upon the RBC shape and volume heterogeneity. This last point will require a rather exhaustive investigation.

These results point to the possibility of directly recording RBC counts with the apparatus described plus a relatively inexpensive curve integrator and a properly calibrated pulse recorder or commercial counter.

### *Hemoglobin Determination*

Both hemoglobin solutions and RBC suspensions present several strong absorption bands in the visible spectrum. However, the strongest band, centered at 425  $m\mu$ , is not suitable for colorimetric analysis, since it falls in the region of anomalous refractive dispersion, as shown by Drabkin and Gordy.<sup>70</sup> Drabkin reviewed this matter more recently<sup>71</sup> and found the weaker band at 550  $m\mu$  to be of almost the same strength in the hemoglobin solutions and in circulating RBCs. To test the performance of the apparatus with this rather delicate system, fresh, oxalated blood was hemolyzed in 0.1 *N* HCl solution and diluted as shown in TABLE 7, where it is taken into account that the apparatus introduced a final dilution of 0.6:1.0. The measurements were made at 520  $m\mu$ . The recorded data finally were converted to the corresponding absorb-

ance values  $A_s$ , the diluent fluid being used as a blank to adjust the instrument's scale span. The results are shown in TABLE 7 along with a set of calibration values reported for the clinical Weston-Rouy colorimeter at 490  $m\mu$ , which are shown for comparison.

In both instances the representative plot is a straight line obeying Beer's law rather satisfactorily. Again, no deviation attributable to the proportioning pump system can be detected.

#### SUMMARY AND CONCLUSIONS

The impact of automation upon biomedical technology urges ever faster acquisition of laboratory data. The financial importance of automation stems from the social and demographic conditions created by generally expanding economies, growing populations, and rapid social transformations. The slowness of primary laboratory measurements, aside from the interrelated problems

TABLE 7  
ABSORBANCY VERSUS HEMOGLOBIN CONCENTRATION MEASURED  
WITH TWO INSTRUMENTS

Hemoglobin concn. (gm. %)	Absorbancy against water	
	Measured at 520 $m\mu$	Reported for Weston-Rouy instrument at 490 $m\mu$
2.42	0.073	0.026
3.36	0.094	0.055
4.86	0.142	0.108
6.55	0.183	0.167
9.68	0.284	0.223
14.54	0.434	0.320
19.36	0.569	0.428

of data reduction and conversion, is still a deterrent in specific, timely data acquisition. A systems-engineering evaluation of the field indicates that at least eight factors must be considered in equipment design.

The analytical apparatus designed includes three basic modules: the fluid-processing, measuring, and programming systems. It utilizes a continuous carrier stream into which metered samples and reagents are injected at controlled intervals. Reactions proceed in transit. Normalization in this dynamic system leads one to replace measurement dimensions of volume by time, duration by distance, and concentration by equivalent rate of mass flow, all of which are time-dependent variables controllable by cam timers and relatively simple implements. The fluid-processing system incorporates a plurality of plug-in units, including special peristaltic pumps, nonturbulent mixers, a synchronous drive, and a unique automatic sampler. These components form expansible arrays of series-parallel combinations capable of performing rather intricate chemical operations.

The measuring system includes a photoelectric colorimeter with automatic remote selection of narrow band-pass filters and scale span adjustment. It is operated conjointly with a special drift-free amplifier and a recording strip-

chart potentiometer. Provisions are incorporated for the scanning of other transducers across the amplifier, with the same benefit of fully automatic scale setting. The conditions set forth for reliable, accurate, fully automatic colorimetry are very stringent and preclude balanced circuits.

The programmer coordinates the functions of the fluid-processing and measuring systems. It incorporates a set of electric cam timers of the lock-and-reset type and operates by short pulses originating in the closing of contacts on a remote keyboard. Operation may be manual or triggered by external automatic machinery. Random programming of any of the analytical procedures available is possible, for any one of the processes can be started while a previous analysis is still in progress.

Conflicting requirements are placed upon the amplifier. The instrument built is of the chopped input type, but it can operate at either low or high signal impedance, as required. A rejection factor of over 1000 and an open-loop voltage amplification of over  $10^8$  were obtained, as well as a satisfactory time constant and good linearity.

Dynamic analysis of the performance was approached from the standpoint of communication theory. The low-pass cut-off frequency of the "linear noisy channel" was considered in determining the "optimum spatial distribution" of analytical samples. Optimum operating conditions were derived by relating the above quantity to the over-all fluid mass transfer rate and to the time constant of the read-out device by using the Sampling Theorem. The speed of operation is limited by initiation of either relaxation, hunting, spurious resolution, or by a state of resonance in the amplifier-recorder system. Such an approach to performance analysis appears valuable for the adaptation of this analytical system to plant stream control.

The apparatus may be adapted for volumetric analysis and may be used with a variety of transducers. However, detailed performance tests thus far have involved chiefly colorimetric analyses, including chromate colorimetry, urine albumin determination by turbidimetry, hemoglobin determinations, red blood cell count, and urine glucose analysis by an improved and rather complex aerodehydrogenase method. The results demonstrate the reliability of the fluid-processing system, the measurement accuracy for the biochemical applications considered, and the versatility of the entire instrumentation scheme.

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# CONTINUOUS AUTOMATIC CHEMICAL ANALYSIS *IN VIVO*

## I. AN INSTRUMENTAL APPROACH TO CHEMICAL PHARMACOLOGY *IN VIVO*

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An instrumental system for continuous automatic colorimetric analysis has been described previously.<sup>1,2</sup> The system is controlled by a multichannel proportioning pump that delivers fixed volume ratios of sample and reagents to other components in the system. Purification of the constituent to be analyzed is effected by continuous dialysis and, after appropriate chemical treatment, optical densities are measured in a ratio-recording dual-beam colorimeter fitted with a flow cuvette. The system can analyze different samples on a continuous basis or continuously analyze or monitor changes in the same sample.

The latter capability suggested its use for the continuous analysis of blood constituents in the intact animal. Thus, the effect of pharmacological agents on blood constituents could be assessed continuously, or the concentration of such agents could be estimated on the same basis. Many similar applications are also possible. The first experiment, carried out to demonstrate the utility of the technique, was the continuous evaluation of the effect of intravenous insulin on blood glucose in the intact rabbit.

### *Experimental*

A standard AutoAnalyzer\* was used. Hoffman's method for the microdetermination of blood glucose<sup>3</sup> was modified suitably for use in the AutoAnalyzer.

*Reagents.* Sodium cyanide (0.5 per cent) and 1 per cent potassium oxalate in 0.9 per cent aqueous sodium chloride was prepared by the following method: dissolve 9.0 gm. sodium chloride, C.P., in 500 ml. distilled water; add 5.0 gm. sodium cyanide, C.P., and 10 gm. potassium oxalate, C.P., and shake until dissolved. Dilute to 1 l. with water. Store in an amber bottle.

Alkaline 0.035 per cent potassium ferricyanide in 0.9 per cent aqueous sodium chloride solution was made up as follows: dissolve 9.0 gm. of sodium chloride, C.P., in 500 ml. of distilled water; add 0.35 gm. finely powdered potassium ferricyanide, C.P., and shake to dissolve. In another flask, dissolve 20.0 gm. of sodium carbonate, C.P., in 100 ml. of distilled water. Add this solution to the potassium ferricyanide solution and dilute to 1 l. with water.

\* Technicon Instruments Corp., Chauncey, N. Y.

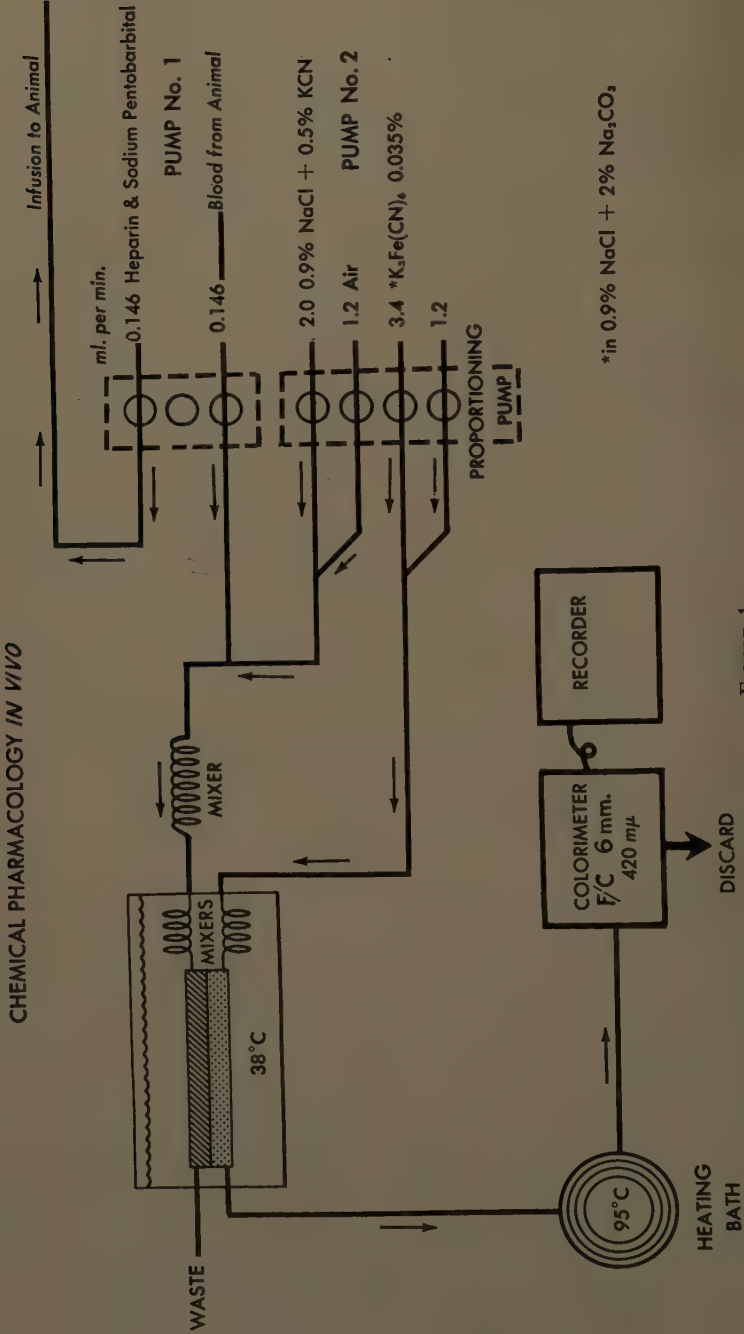


FIGURE 1



*Flow diagram.* The flow diagram for the continuous automatic determination of glucose is seen in FIGURE 1. The numbers adjacent to the tubing in the proportioning pump refer to milliliters of fluid or air pumped per minute. The stream of blood from the animal is diluted initially and segmented by air by mixing with a stream of segmented sodium cyanide reagent. The diluted stream is sent through the constant-temperature dialyzer at 38° C., where it is dialyzed against a concurrent stream of segmented potassium ferricyanide reagent. In this step a portion of the glucose and potassium cyanide diffuse across the membrane. When the diffusate stream emerges from the dialyzer, it is passed into a 95° C. thermoregulated heating bath equipped with a 5-min. time delay coil. During this treatment, the ferricyanide is reduced in propor-

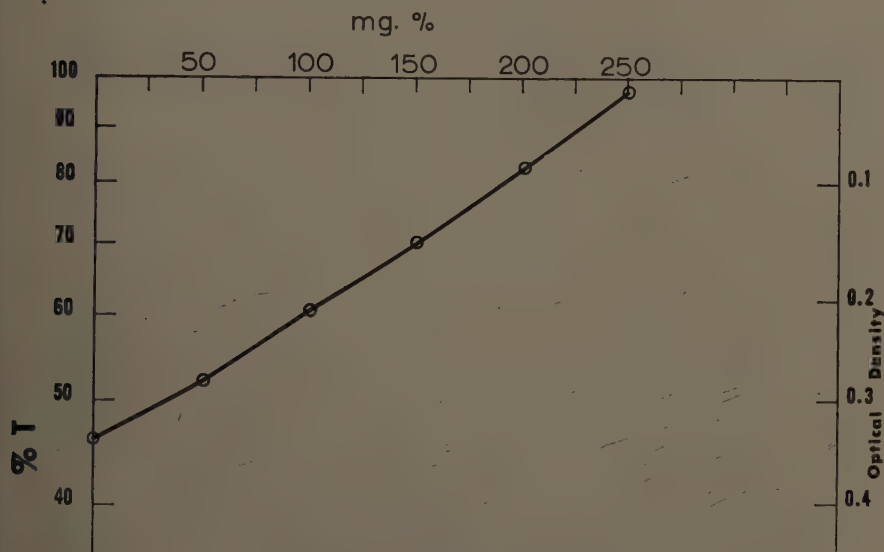


FIGURE 2

tion to the concentration of glucose present. After this step the stream is passed into the colorimeter where the per cent transmittance is measured at 420 m $\mu$ . A typical standard curve obtained with this method for standard aqueous glucose solutions is seen in FIGURE 2. Continuous glucose monitoring of heparinized blood samples for up to 9 hours by this method has shown that the deviation from the mean was less than  $\pm 1$  per cent.

*Surgical preparation of animals.* Male adult Belgian hares that had been fasting for 10 hours were used. The hair on the ventral part of the neck was shaved mechanically and the skin was cleansed with tincture of Merthiolate. Initial sedation was induced with sodium pentobarbital (35 mg./kg.) administered through a marginal ear vein approximately 15 min. before surgery was begun. The animal was then fixed to the working surface by leg clamps. An incision was made from a level just below the mandible to the sternum clavicular. The soft tissues and muscles were dissected carefully to expose the jugular

and the various collateral veins. The bifurcation of the external and internal jugulars was freed of all soft tissues. Six nylon sutures were passed around the external jugular. Natural polyethylene tubing (medical grade) of 0.023-inch inside diameter, cut in lengths of approximately 30 inches, siliconized

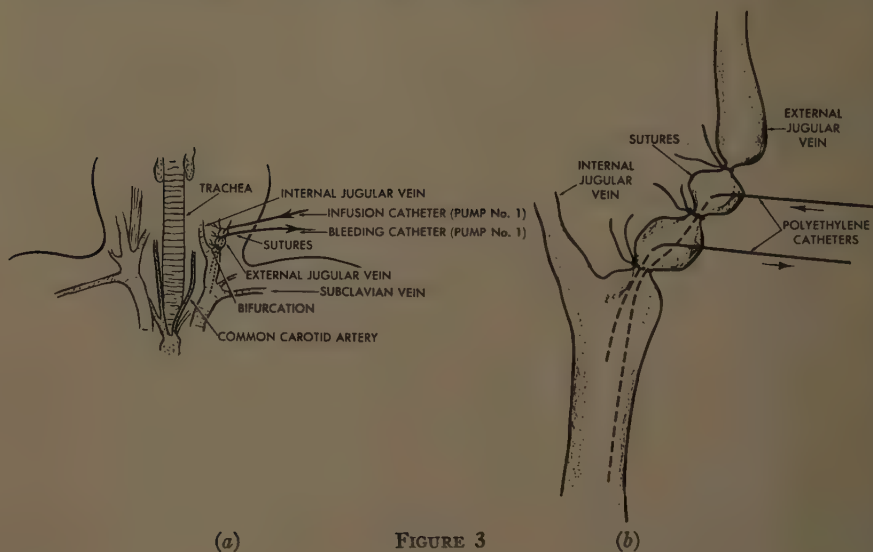


FIGURE 3

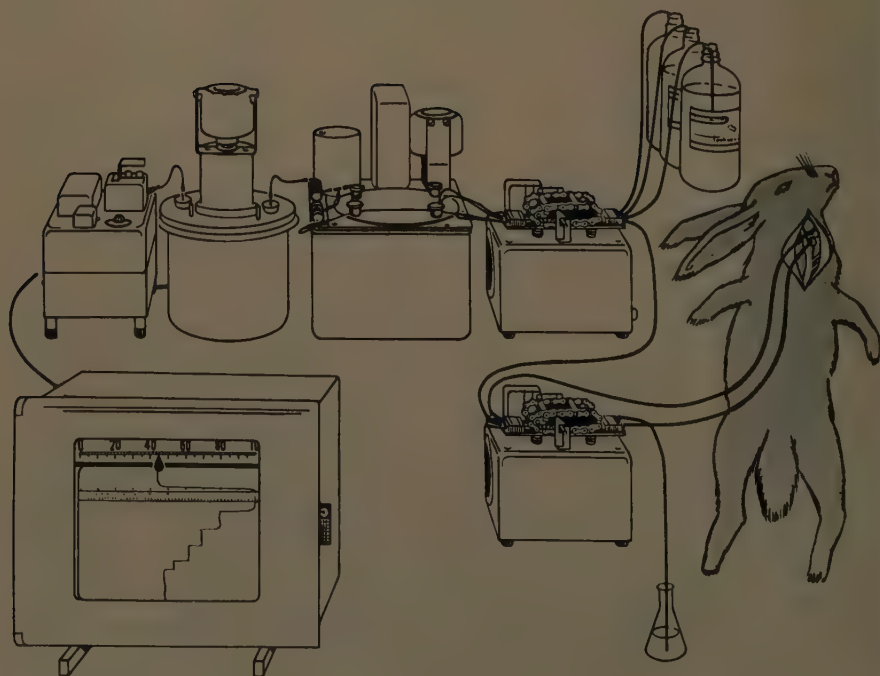
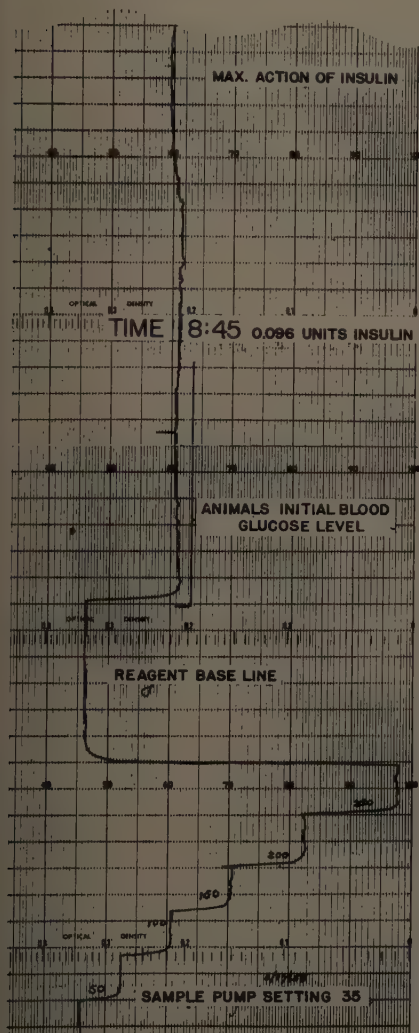
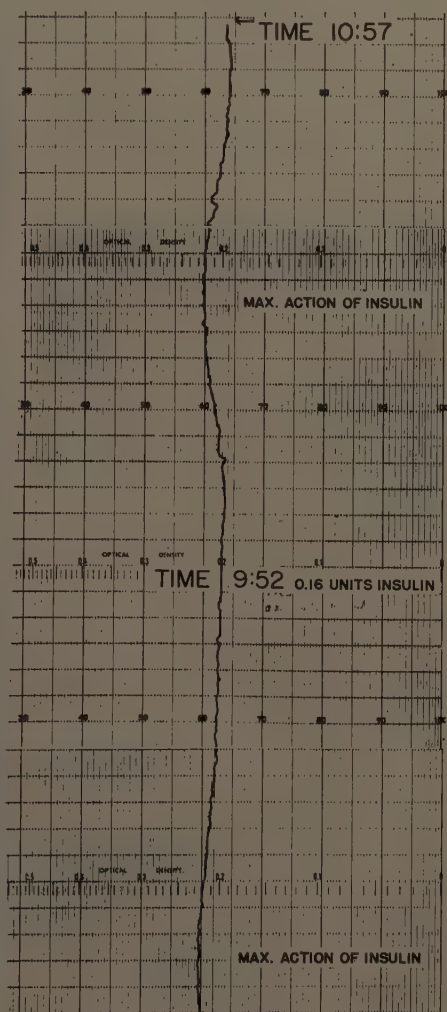


FIGURE 4

with Siliclad,\* and heat-sterilized, served as catheters. Each was placed inside a 17-gauge needle to a level just above the point. The external jugular was pierced with the needle about  $\frac{1}{4}$  inch above the bifurcation, and the cath-



RECORDING



RECORDING (Cont')

FIGURE 5

eter was pushed below the level of the puncture. Sutures immediately below and above the puncture were tightened and the needle was removed while the catheter was held in place. A suture was then tightened around the catheter to prevent seepage of blood. A second catheter was inserted between the first and the bifurcation by the same technique. Details of the surgically

\* Clay-Adams, Inc., New York, N. Y.

prepared veins are seen in FIGURES 3a and b. The first or anterior catheter is used for the continuous removal of blood; the second, for the administration of such additives as sedative, anticoagulant, and medication. In this way, dilution of the blood sample with infused agents is avoided.

The anterior catheter is connected through a variable speed-proportioning pump to the sampling side of the AutoAnalyzer manifold as shown in FIGURE 1. The pump is set to deliver blood at 8.8 ml./hour (0.146 ml./min.) through the catheter. A second channel of the pump is used for the continuous infusion of sedative and anticoagulant at the same rate. An aqueous solution containing 2.5 mg./ml. sodium pentobarbital and 10 U./ml. sodium heparin is used for this purpose. The infusion and blood sampling are started as soon as the catheters are in place. This technique produced adequate sedation and heparinization for the 9-hour duration of the experiment.

A generalized view of the over-all experimental setup is seen in FIGURE 4.

### Results

The continuous recording of the initial blood glucose level of the anesthetized animal is shown in the chart in FIGURE 5. The level remained at approximately 110 mg. per cent for the initial 30-min. period. At this time, 0.096 U. crystalline insulin was infused rapidly through the posterior catheter, which was momentarily disconnected from the barbiturate-heparin stream for this purpose. There was no observable effect for about 15 min. Then the glucose level gradually declined to a maximum depressed level at 95 mg. per cent at 45 min. after introduction of insulin. It then began to rise and, after another 45-min. period, had returned to a stabilized level at approximately 125 mg. per cent. This stabilized level was observed for 45 min., and a second dose of insulin, 0.16 U., was infused as previously described. Again no change in glucose level was noted for about 15 min. After this time, the level began descending and reached a minimum at 95 mg. per cent 30 min. later. It then began a gradual ascent to a maximum level at 120 mg. per cent during the next 45 min. This level was maintained by the animal for 40 min. At this point, the experiment was discontinued. The catheters were removed and the incised area was treated with a powdered mixture of streptomycin and penicillin. The incision was closed with clamps.

The previous experiment demonstrates the applicability of continuous automatic analysis to the study of chemical pharmacology or pharmacodynamics *in vivo*. The initial experiments were limited to continuous analysis of a single constituent.

## II. AUTOMATIC AND CONTINUOUS *IN VIVO* ORGAN PERUSAL EXPERIMENTATION

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It became apparent from the foregoing application that one could apply such a technique to continuous monitoring of the influence of a single organ on



the composition of body fluids. From this it followed logically that any substance altering the physiological integrity of the organ would in turn alter the composition of these fluids. Such physiological changes could be detected by monitoring efferent and afferent blood vessels of body organs and the excreted and secreted products of these organs. It could also be applied to studies of the metabolic fate of drugs or the metabolic changes in an organ under the influence of a drug in contrast to changes occurring through normal metabolic processes.

For the preliminary work in organ perusal, the metabolism of the kidney as affected by the administration of diuretics was studied by simultaneously analyzing total carbonates in the renal artery and vein and in the ureter.

### *Methodology*

The method used for the determination of total carbonates is that developed by L. T. Skeggs for use with the following AutoAnalyzer reagents: (1) acid diluent, consisting of 2.8 ml. concentrated sulfuric acid and 1 ml. Dow-Corning Antifoam B, made up to 1 l. with distilled water; (2) bicarbonate-carbonate buffer, containing 2 parts 1.0 *M* sodium bicarbonate to 1 part 1.0 *M* sodium carbonate; (3) the color reagent, made with 1.25 ml. carbonate-bicarbonate buffer and 3 ml. 1 per cent methanolic solution of phenolphthalein diluted to 1 l. with distilled water; and (4) sodium hydroxide wash solution, 40 gm. sodium hydroxide to 1 l. water.

The flow diagrams for the continuous analysis at the 3 parameters (measurement locations) are shown in FIGURES 6*a* and 6*b*. The numbers adjacent to the tubing in the proportioning pump refer to the milliliters of fluid or air pumped per minute. The stream of blood from the animal is heparinized initially *in situ* at the cannula tip and subsequently mixed with dilute sulfuric acid and segmented by CO<sub>2</sub>-free air. The combined streams are then mixed in a double-length mixing coil and diverted to a trap where the CO<sub>2</sub>-enriched air phase is separated from the liquid stream. An aliquot of the CO<sub>2</sub>-enriched air phase collected in this trap is aspirated at a constant rate through the pump and used to segment a stream of a weakly alkaline buffered solution of phenolphthalein. The carbon dioxide gas is absorbed by the alkaline solution; a decrease in *pH* is reflected by a color change in the indicator. The resultant streams are then diverted to a 6-mm. flow cuvette (10-mm. cuvette for urine analysis) and all are read at 550 *mμ* and the per cent transmittances recorded.

### *Preliminary Preparation of the Animal*

A male adult dog weighing 11.6 kg. was fasted for 24 hours prior to the experiment. One-half hour before the animal was anesthetized for surgery, it was hydrated with 60 ml./kg. tap water. The hair on the forearms and in the dorso lumbar area was shaved. The animal was anesthetized with an initial dose of 30 mg./kg. pentobarbital intravenously and was maintained in sedation throughout the experiment with an infusion of 3 mg./kg./hour pentobarbital in a 5 per cent mannitol solution. The 5 per cent mannitol infusate, administered at a rate of 2.9 ml./min. through a polyethylene catheter inserted in the arm vein, was used to maintain a good urine flow and to keep the dog

in water balance. The bladder was catheterized and drained into a graduated cylinder where the volume excreted could be observed.

To facilitate the surgical technique and obviate the need for rib retraction, the left kidney was chosen for the study. The operative area was cleansed with soap and an antiseptic solution was applied. An incision approximately

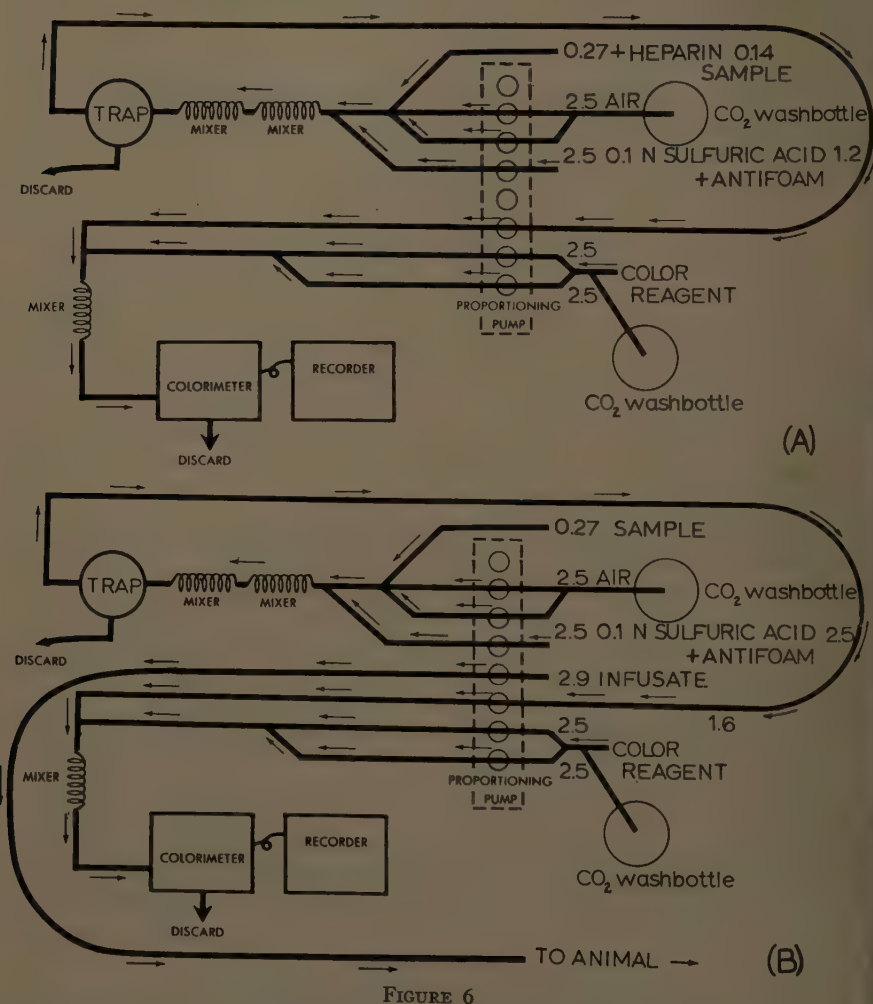


FIGURE 6

15 cm. in length was made from the lumbar muscles ventrally in conformation with and parallel to the last rib. The muscles were separated as much as possible by blunt dissection, and all bleeding vessels were ligated with silk ligatures.

The retroperitoneal space and the left kidney were exposed by retraction. The kidney was retracted ventrally and the renal artery, vein, and ureter were exposed and isolated by blunt dissection from the perirenal fat.

The ureter was ligated and excised. A polyethylene catheter of a bore large enough to permit free flow of urine was passed into the isolated, exposed ureter to a point just below the renal pelvis. Ligatures were passed around the ureter and tied to secure the catheter in place. The end of the catheter protruding from the ureter was attached to one arm of a T connection. Inserted into the opposite arm was the polyethylene sampling tube that carried the urine sample to the analytical system. Attached to the leg of the T was an overflow drainage tube to carry off excess urine to a graduated cylinder (FIGURE 7).

The isolated renal artery was occluded with a bulldog clamp to stop circulation through the kidney. The isolated renal vein was clamped at close proxim-

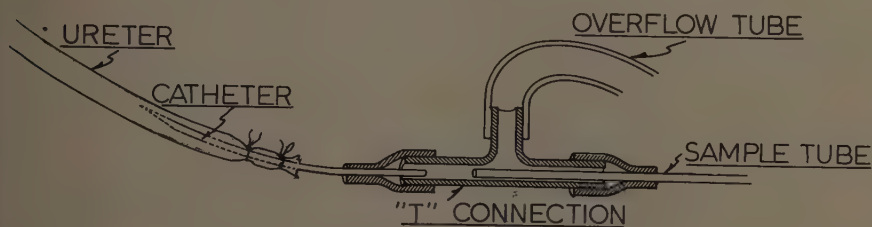


FIGURE 7.

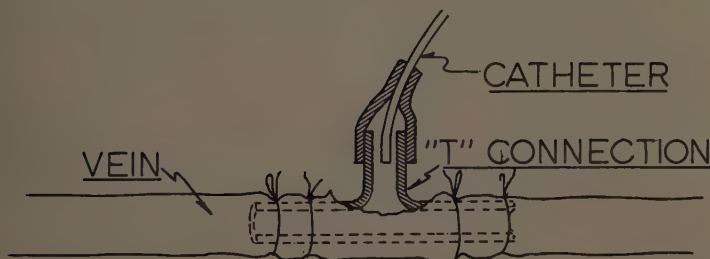


FIGURE 8

ity to the kidney and at the junction with the posterior vena cava. A longitudinal incision of sufficient length to admit a T connection was made in the renal vein between the two clamps. The T tube was secured with double ligatures of surgical silk placed around both arms of the T. The leg of the T connection held the double cannula catheter, the tip of which was placed just at the edge of the blood stream flowing through the portion of the T simulating the vein (FIGURE 8).

An area over the upper femoral canal of the right leg was shaved and cleansed. An incision approximately 5 cm. long was made over the canal. The femoral artery was exposed and isolated by blunt dissection. With the aid of bulldog clamps and silk sutures a double cannula catheter was inserted and passed up the femoral artery into the aorta so that the tip of the catheter rested approximately in the area of the renal arteries. The catheter was then secured with the ligatures.

Both incisions were closed with interrupted sutures of 00 surgical silk in the muscle and by Michel's clamps in the skin.

### *Difficulties Met in Surgical Techniques*

In our first preparations we attempted to insert the catheters through a small incision made in the walls of the artery and vein and to secure them with purse-string sutures of 50 cardiovascular silk. However, we found this to be unsatisfactory, since it created a distortion of the vessel walls and permitted seepage of blood around the catheters and through the suture holes; in some cases it induced clot formation in the vessel. We also had difficulty in securing the catheters well enough to prevent them from shifting their positions. Therefore, we resorted to the technique described previously, which proved successful.

The renal vein must be prevented from kinking against the rigid ends of the T tube, which would result in an obstruction of the blood flow out of the kidney.

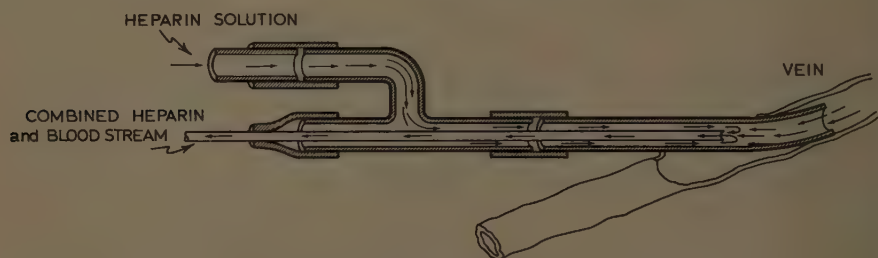


FIGURE 9

The ligatures holding the T must be sufficiently tight to prevent leakage of blood.

The T should be filled with heparin from the onset, to prevent blood clotting while it is being inserted into the vein. The tip of the double cannula catheter should be just at the edge of the blood stream to avoid increased turbulence and to minimize the possibility of clotting.

FIGURE 9 shows a cross-section of the plastic double cannula catheter inserted in a blood vessel. Insertion of the double cannula directly, sans T connection, through a collateral blood vessel into the main vessel is an alternate approach that has been used successfully. FIGURE 10 illustrates another approach that utilizes T's at all 3 parameters. The flow of 0.25 per cent heparin or anticoagulant is fed to an H fitting that permits the solution to flow between the walls of the inner and outer cannula to the tip, where it is mixed with the in-flowing stream of blood. Since the volume of the heparin stream is smaller than the total volume aspirated by the sample tube, the anticoagulant is prevented from entering the blood stream of the animal. The inner cannula is connected through the H fitting to the sample pump line. The idea of a double cannula was conceived initially by Duncan Holaday.\*

\* Department of Anesthesiology, University of Chicago Clinics, Chicago, Ill.



The disposition of the equipment, including units for sampling, infusing, and analyzing, may be seen in FIGURE 11. It will be noted that the samples from all 3 sites are handled through a multichannel pump of variable pumping rate. The same pump was used to deliver the heparin solution to the arterial and venous cannulae. The infusion of the sedative in 5 per cent mannitol was accomplished through a pump tube on one of the proportioning pumps handling the analytical phase. It will be noted in FIGURE 11 that a separate

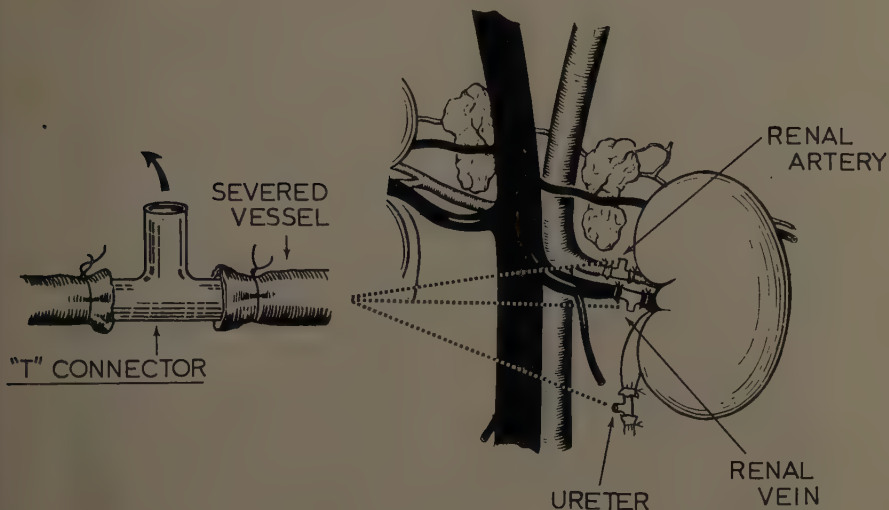


FIGURE 10

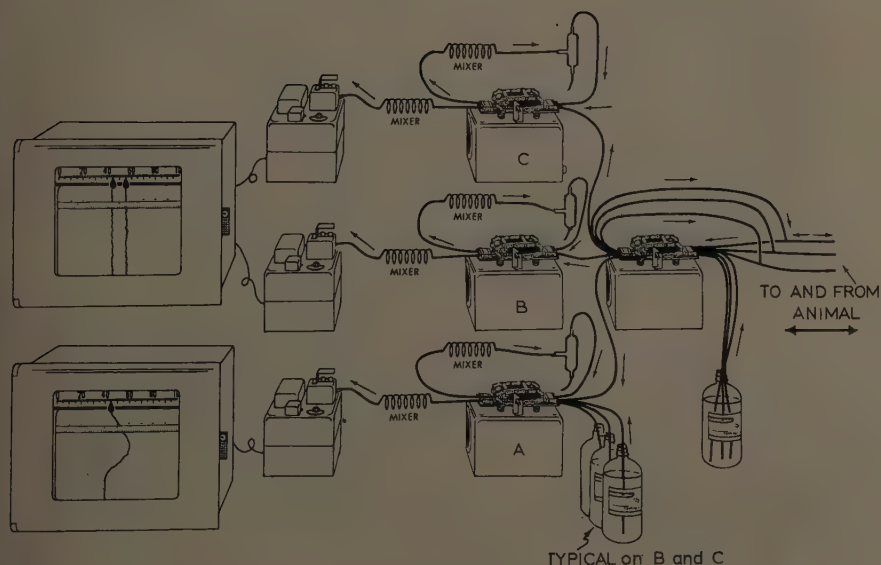


FIGURE 11



FIGURE 12

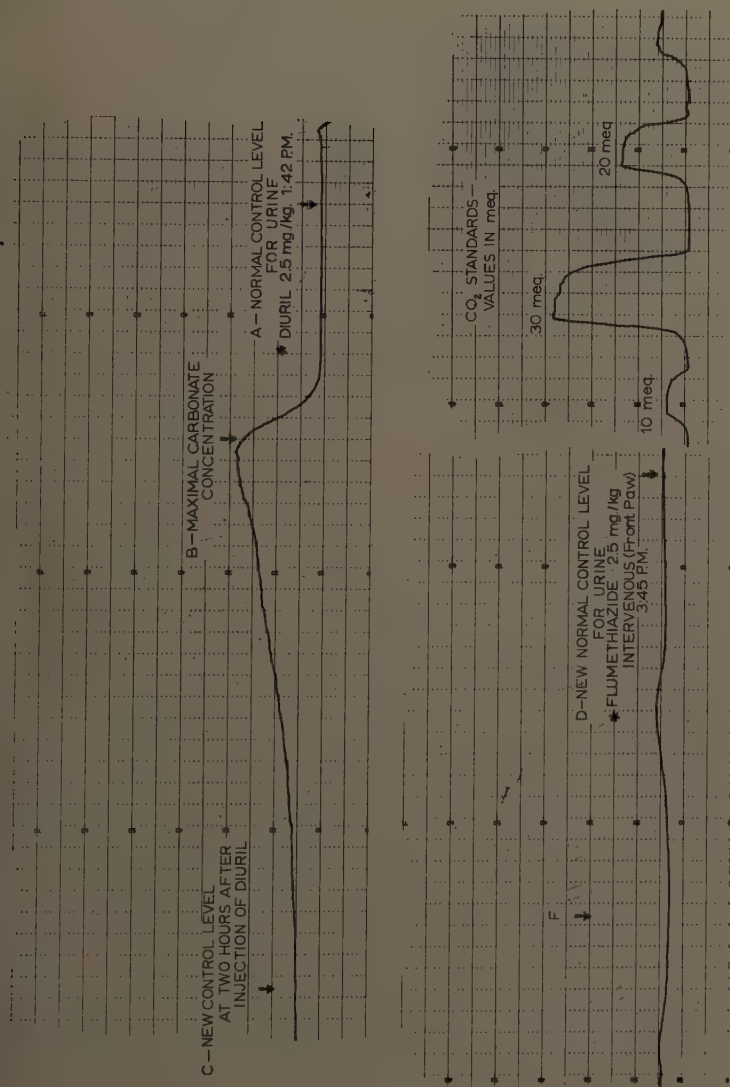


FIGURE 13

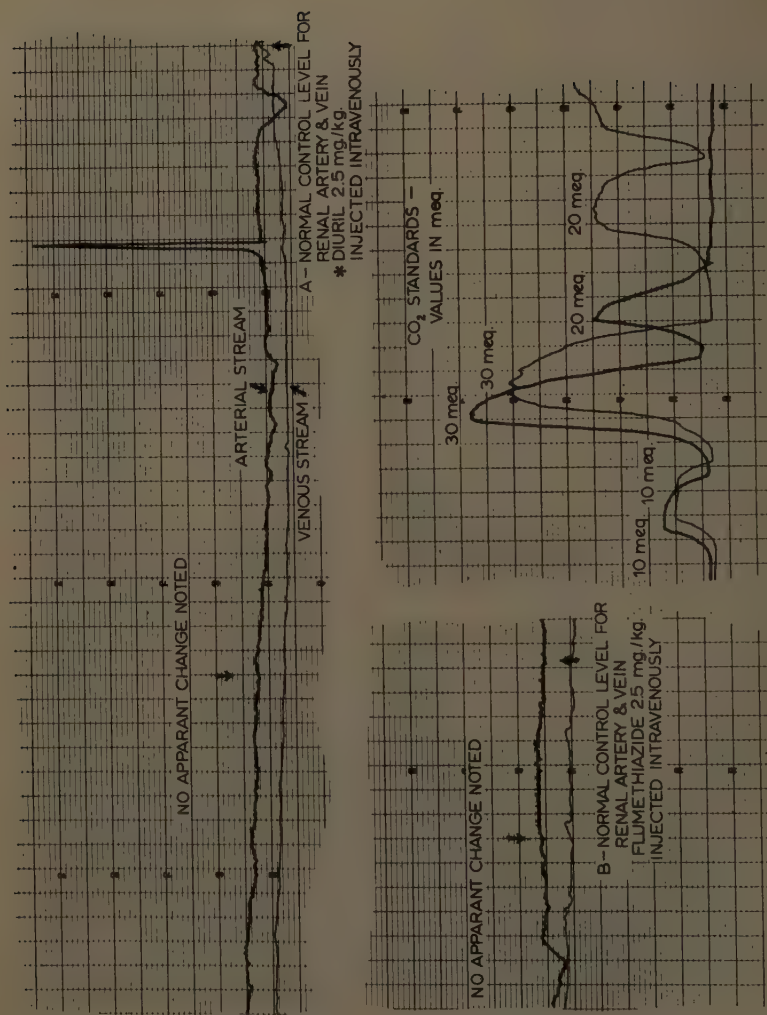


FIGURE 14



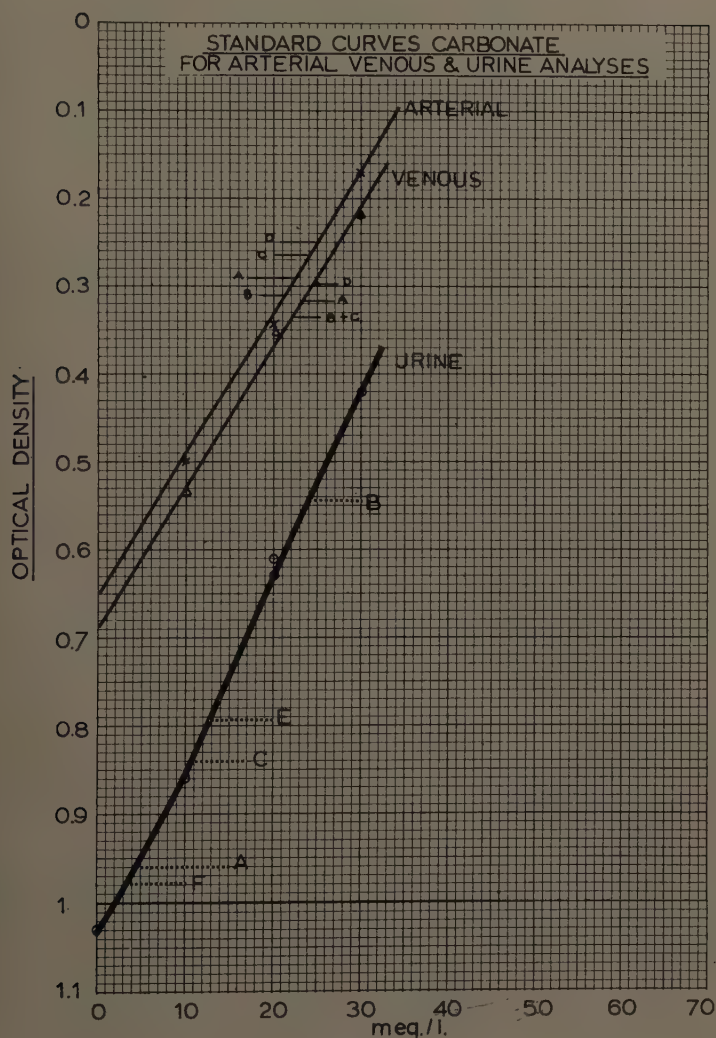


FIGURE 15

TABLE 1  
CONTINUOUS AUTOMATIC ANALYSIS OF BICARBONATE *IN VIVO*

Substance	(Mg./kg.)	Maximal bicarbonate concentrations			Maximal changes in bicarbonate concns.			Maximal effect (min.)
		Arterial (mEq./l.)	Venous (mEq./l.)	Urine (mEq./l.)	Arterial (mEq./l.)	Venous (mEq./l.)	Urine (mEq./l.)	
Control (A)	—	23.0	23.0	4.5	—	—	—	—
Chlorothiazide (B)	2.5	22.0	22.0	24.5	-1.0	-1.0	+20.0	10
Control (C)	—	24.6	23.0	10.7	—	—	—	—
Flumethiazide (D)	2.5	25.5	24.5	12.8	+0.9	+1.5	+2.1	6

analytical system was used for each parameter. FIGURE 12 illustrates the disposition of the equipment under actual experimental conditions. The results of the analysis from the arterial and venous parameters were recorded on a dual-pen ratio-recording system. The result of the urine analysis was recorded on a separate single-pen ratio-recording system.

### Results

The recorded data show that the most dramatic changes in carbonate concentration occurred in the urine when 2.5 mg. per cent per kilogram of chlorothiazid was administered intravenously. FIGURE 13 shows that a maximal effect occurred 10 min. after the injection of the drug (point B). Two hours after the injection of the chlorothiazid, the urine established a new control level (point C). At this time 2.5 mg./kg. of Flumethiazide was administered intravenously (point D). In contrast to chloroderivative, Flumethiazide had little effect upon the excretion of total carbonates (point E). It was observed from the continuously recorded data that a depression of the total carbonate level also occurred with this drug following the slight initial elevation (point F).

The continuously recorded data for the arterial and venous streams showed no apparent change with the administration of either drug (FIGURE 14).

FIGURE 14 also shows the recorded standard. FIGURE 15 shows the constructed standard carbonate curves from which the arterial, venous, and urine levels were calculated. TABLE 1 shows a composite picture of the effects observed.

### Conclusions

The experiments demonstrate the applicability of continuous automatic analysis *in vivo* to the study of blood and body fluid chemistry as influenced by the body as a whole or by an individual organ. The results also indicate that this technique could have a place in primary as well as advanced pharmacological studies and in research of a physiological or biochemical nature. In addition, by means of multiple AutoAnalyzers or components thereof, several different physiological constituents or levels of pharmacological agents can be assessed simultaneously on a continuous basis. The approach is limited only by difficulties that will be encountered in surgical techniques and methods of analysis.

### ACKNOWLEDGMENT

We are indebted to John Poutsika and Lloyd Milstein of the Squibb Institute for Medical Research and to George Macduff and Alvaro Ferrari, Research Laboratories, Technicon Instruments Corporation, for valuable suggestions and assistance during the course of the work.

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# THE USE OF THE AUTOANALYZER IN STUDIES OF THE PHYSIOLOGICAL CHEMISTRY OF THE PAROTID GLAND\*

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## *Introduction*

The mechanism of passage of the chemical constituents of saliva through the salivary gland cells is not understood. It has been demonstrated that some substances seemingly are excreted according to laws of simple diffusion while, with others, the gland cells seem to exhibit specific selectivity in permeability. Controversy also exists concerning the possible reabsorption of materials from the duct systems of the glands, and the possibility that the duct cells may themselves contribute to the saliva by actively secreting materials into the tubular lumen.

It can be stated without equivocation that saliva is not an ultrafiltrate of blood, that is, that mechanisms are active within the glands that markedly alter salivary constituent levels from those found in the blood. Salivary potassium levels are approximately 5 to 6 times those found in blood serum, while the sodium concentration in saliva is approximately one fifth that found in serum; chlorides of saliva are present at only about one fifth the serum level; urea in saliva is 75 to 90 per cent of that found in serum; glucose has been thought to be totally absent from salivary secretions; total protein content of parotid fluid is usually less than one tenth that found for serum; inorganic phosphate levels in parotid fluid are 2 to 3 times those of serum; and calcium is present in parotid fluid in less than one half the serum concentration.

An exceedingly important consideration in the interpretation of results of chemical analyses of saliva is that the concentrations of some of its chemical constituents are influenced by the rate of flow of saliva from the glands. Sodium, chlorides, calcium, and total protein, it is generally agreed, are positively correlated with flow rate, that is, the more rapid the flow, the greater the concentration of these variables per unit volume. We find that urea exhibits a negative correlation with flow rate, while potassium seems to be independent of flow rate.

Much of the research effort in our laboratories is directed toward the evaluation of possible indices of adrenocortical status. Although we were interested primarily in parotid fluid electrolytes, we have found since that human parotid fluid (and whole saliva) invariably contains a measurable amount of free 17-hydroxycorticosteroids and, more important, that the parotid fluid concentration varies directly with the serum level. We have substantiated this finding by the oral administration of synthetic analogues of hydrocortisone<sup>1</sup> (prednisolone, triamcinolone, Decadron, and Medrol) and by the intramuscular injection<sup>2</sup> and intravenous infusion<sup>3</sup> of ACTH. In all instances, changes in the steroid content of parotid fluid have paralleled the serum changes. Thus

\* The contents of this paper represent the opinions of the authors and are not to be construed as representative of United States Air Force policy.

we are provided with a completely nonstress-producing way of continuously evaluating adrenocortical status in humans.

Along with steroid studies, a battery of supportive determinations is usually carried out on our parotid fluid samples. Since the 17-OHCS analysis requires 5.0 ml. of undiluted fluid, one of our primary concerns is the conservation of sample in other procedures and the maximum utilization of diluted samples when feasible. It is with the adaptation of the AutoAnalyzer,<sup>4-6\*</sup> a continuous flow, automatic recording colorimetry system, to 4 of these supportive analyses—calcium, inorganic phosphate, urea nitrogen, and glucose—that this paper is concerned.

In the determination of actual concentrations of variables, we have avoided interpretation from standard curves and have substituted mathematical calculations for them. Calculation has been facilitated by linearization of the recorder response when practicable and, for the semilogarithmic recordings, by the conversion of per cent transmission readings to optical density.

In the statistical analysis of the performance of the various methods on replicates, the standard deviation has been used as the expression of precision. In all instances the standard deviation represents the square root of the sum of the squares of the differences between duplicates ( $x_2 - x_1$ ) divided by 2 times the number  $n$  of duplicate pairs, or

$$\text{S.D.} = \frac{[\sum (x_2 - x_1)^2]^{1/2}}{2n}$$

The coefficient of variation is the percentile ratio of the S.D. to the mean:

$$\text{S.D.} \cdot 100 / \text{Mean} = \text{C.V.} \quad (\%)$$

### *Salivary Calcium*

Throughout the years, the determination of calcium has been based upon oxalate precipitation procedures. The majority of these methods employed the Kramer-Tisdall<sup>7</sup> method (1921) as modified by Clark and Collip<sup>8</sup> in 1925, or other procedures that varied only slightly from it. Schwarzenbach and his group<sup>9</sup> provided basic contributions in complexometric analysis by studying the chelation of divalent and trivalent ions by EDTA. In recent years several indicators, namely, murexide,<sup>10</sup> Calcein,<sup>11</sup> Cal-red,<sup>12</sup> Calcon,<sup>13</sup> and Plasmocorin B<sup>14</sup> have been offered as specific in calcium determinations.

Virtually all salivary calcium studies have depended upon the precipitation of calcium as the oxalate with measurement by a titration procedure. Flame photometry for salivary calcium analysis is unsatisfactory, owing primarily to the wide variation in sodium content of the samples. It is quite easy, by altering the flow rate for a given subject, to vary the sodium content of the saliva from 10 to 80 mEq./l. in consecutive samples. Variations such as this make flame photometric calcium results extremely difficult, if not impossible, to interpret.

Ericsson<sup>15</sup> has applied EDTA titration with Eriochrome Black T indicator to saliva for the determination of combined Ca and Mg. He then determined

\* Technicon Instruments Corp., Chauncey, N. Y.



Mg by the titan yellow procedure. He found the use of murexide for calcium determination impracticable, owing to the weak color shift.

In the AutoAnalyzer calcium method, the sample is mixed with buffered Hyamine reagent ( $pH$  4.0) to free the protein-bound calcium. After dialysis of the sample into water, the buffered murexide indicator is added at a  $pH$  of 11.5, and the color is read at 505  $m\mu$ . This is a modification of the method suggested by Williams and Moser<sup>16</sup> which was, in turn, based upon the original contribution of Schwarzenbach and Gysling<sup>10</sup> on the use of murexide.

With the AutoAnalyzer, parotid fluid calcium may be determined on untreated samples with some degree of flexibility. With the 10-mm. flow cuvette and a baseline setting of approximately 50 per cent transmission, satisfactory determinations may be performed with the machine plotting either linearly or semilogarithmically. With this cuvette it is impossible, however, to depress the reagent base line to the high percentage transmission values that give the desirable maximal peak height production. Peak height is also decreased if the linearized recorder is used. For maximal performance the following procedure is recommended:

With the conventional calcium manifold, parotid fluid is analyzed for calcium at the rate of 20 determinations per hour with no sample spacing necessary. The serum diluent (Hyamine) is made to contain 0.03  $M$  sodium chloride, and the standards are prepared in 0.40  $M$  NaCl. The 6-mm flow cuvette is employed, and the base line, with the machine nonlinearized, is set in the vicinity of 99 per cent transmission. FIGURE 1 demonstrates a typical run of standards from 0.5 to 3.0 mEq./l. Ca under the above conditions. The peak configuration is very acceptable and a linear calibration curve results when optical density is plotted against calcium concentration. It should be added, however, that when the range of this curve is extended upward, a sharp break occurs at 3.0 mEq./l. and that from 3.0 to 5.0 mEq./l. the curve, although linear within itself, has a different slope. This offers no particular difficulty in parotid fluid determinations, since the lower curve is usually adequate for the range.

Earlier studies with serum calcium determinations had made it clear that the amount of sodium chloride in the standards exerted a definite influence upon the final calcium values for the unknown samples. For the parotid fluid calcium method, then, it was necessary that all samples and standards contain sufficient NaCl to mask the effect of any additional NaCl present in individual samples and to provide standard dialysis comparable to sample dialysis. It was found necessary, regardless of the NaCl to be placed in the standards, that some NaCl be added to the sample prior to dialysis in an effort to minimize the effect of NaCl differences between the samples. This was necessary because sodium and chloride in parotid fluid are extremely variable between subjects and, depending upon the rate of secretion of saliva, even between samples from the same individual. With the Hyamine solution made to contain 0.03  $M$  NaCl (the equivalent of 150 mEq./l. NaCl in the sample), the effect of added sodium chloride within physiological limits did not appear significant.

Experiments then were undertaken to determine the amount of NaCl, if any, that it would be necessary to add to the standards in order that the final calcium values be in essential agreement with the results of accepted manual

determinations. A complication arose when it developed that what we had been classifying and measuring as an effect of differing NaCl molarities was, in fact, the effect of differing volumes of specimen in the sample cups. When the NaCl-containing standards were replaced at the end of a run with those containing differing amounts of NaCl and the parotid fluid-containing cups were rerun without being refilled, the results were distinctly different. FIGURE 2 shows the results with 2.0, 1.5, 1.0, and 0.5 ml. samples of each of four concentrations of the  $\text{Ca}^{+}$  standard. These samples were aspirated at the rate of

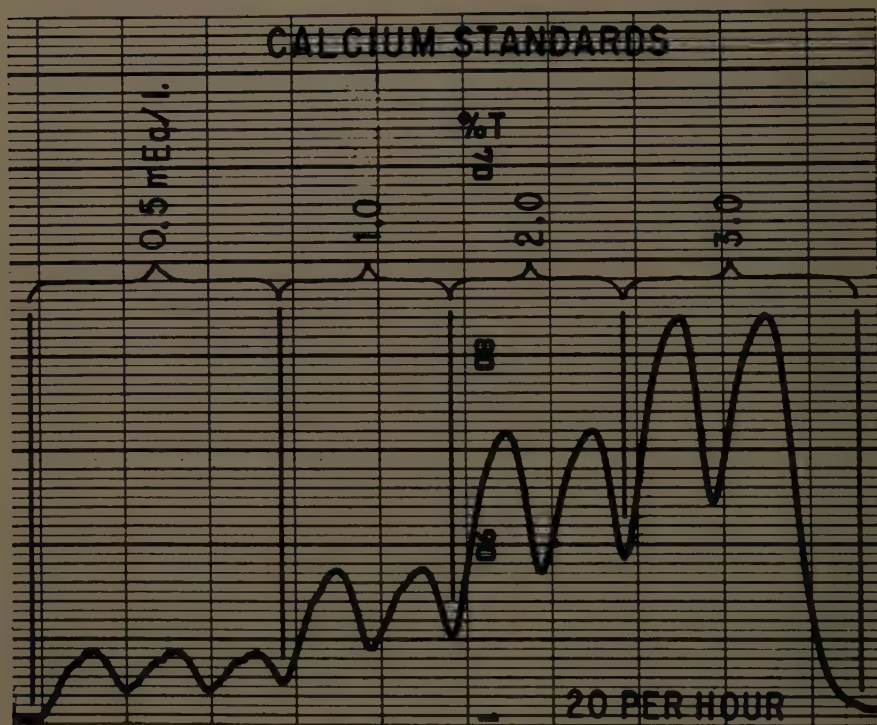


FIGURE 1

40 per hour with water spacing. Variation of the volume in the sample cup did not appreciably alter the readings for the 0.5-mEq./l. standard, but at all higher concentrations there were definite depressions of the peaks, with decreases in sample volume. When the run was repeated at 20 per hour with no spacing, this effect disappeared. A sampling cup was filled with 2.5 ml. of a 2.0 mEq./l. standard, and repeated aspirations were made from this cup. At 40 per hour with water spacing, the first two peaks were quite comparable, the third was a bit lower, and the next three were markedly lower than their three predecessors. The greatest drop occurred between the third and fourth samples, when the theoretical cup content changed from 1.86 to 1.54 ml. When the run was repeated at a speed of 20 per hour with no spacing, color development was

much more complete, and sample volume did not influence the peak as before. It was thus evident that the rate of 40 per hour, even with spacing between samples, was not satisfactory.

The transmission base line of 99 per cent was selected in order to obtain greater peak height. Although the slope of the semilogarithmic plot of the

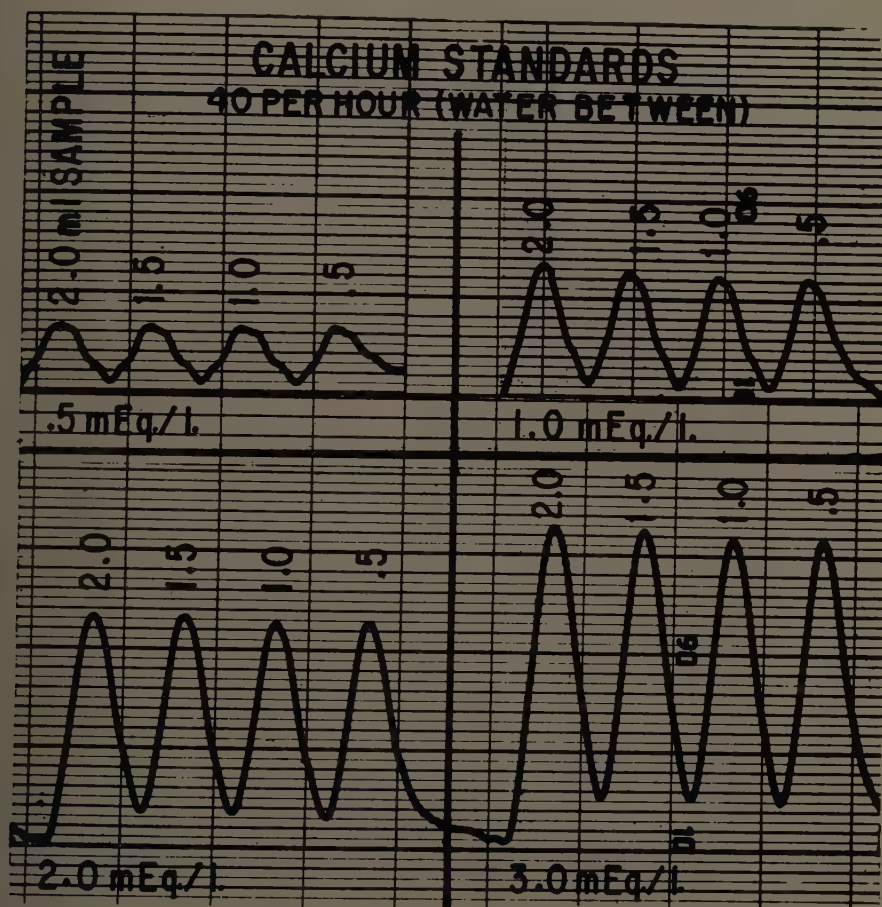


FIGURE 2

standard curve is the same no matter what base line figure is chosen, the peak height varies directly with the per cent transmittance of the base line. The desirability of the selected base line is made clear in FIGURE 3, which shows peaks for a 2.0 mEq./l. standard processed from 8 different base lines.

The amount of NaCl to be added to the standards was determined, as previously mentioned, from tests on a series of pooled parotid fluid specimens against standards containing varying concentrations of NaCl and comparison of these results to quintuplicate determinations by the Clark-Collip<sup>8</sup> modifica-

tion of the Kramer-Tisdall<sup>7</sup> method. A typical experiment is outlined in FIGURE 4. The mean titration calcium value was 1.66 mEq./l. for this pool, which conforms very closely to the result with 0.40 M NaCl in the standards. Throughout the series of studies with parotid pools, results obtained by the manual method agreed closely with the results derived from the 0.40 and 0.50 M NaCl standards, agreement with former standards being slightly superior.

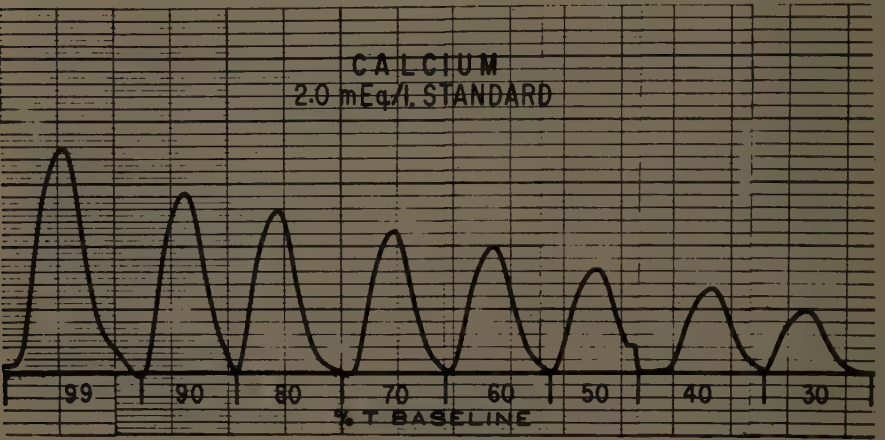


FIGURE 3

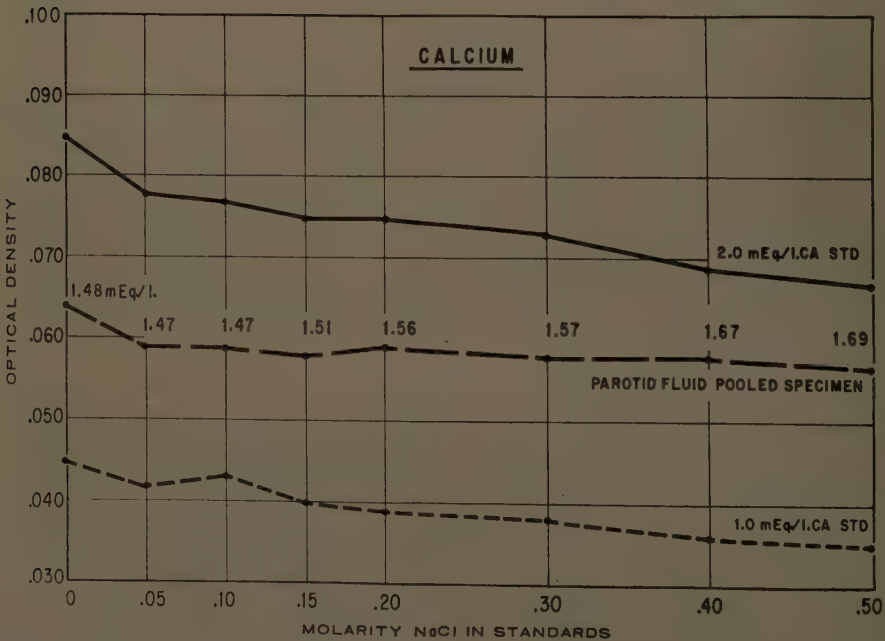


FIGURE 4



It should be reemphasized that the NaCl in the Hyamine is additive to the amount shown on the abscissa and that, even with the standard designated as containing no NaCl, the Hyamine adds the equivalent of 150 mEq./l. of NaCl to both standards and sample.

The recovery of added known amounts of calcium to portions of a pooled parotid fluid specimen is outlined in FIGURE 5. The pool value was calculated at 1.74 mEq./l.; this value was raised to 1.86, 2.09, and 2.32 mEq./l. by measured additions, and the mean recovery was found to be 99.5 per cent.

Forty parotid fluid specimens were processed in duplicate, and the coefficient of variation for the method was estimated to be 4.31 per cent. The mean for the entire series of observations was 1.53 mEq./l. and the standard deviation

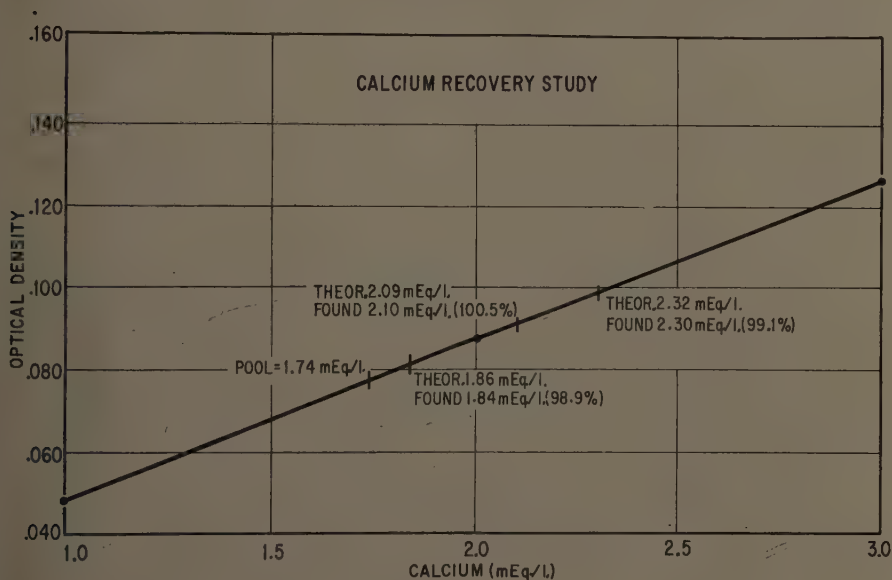


FIGURE 5

was 0.066. Since this 20-per-hour unspaced testing rate had proved superior to the 40 per hour spaced rate, a net rate of 10 per hour—20 per hour with water spacing—was tested to see if further improvement could be effected. Standards were processed by this method, and the standard curve resulting therefrom was virtually identical to that from the 20-per-hour method. A 40-sample duplicate run by this method provided a mean value of 1.55 mEq./l., a standard deviation of 0.073, and a coefficient of variation for the method of 4.71 per cent. Since these results were in no way superior to those obtained with the faster method, the latter was retained.

### *Salivary Inorganic Phosphate*

Virtually all reported salivary phosphate analyses are based upon early contributions to methodology made by Bell and Doisey,<sup>17</sup> Tisdall,<sup>18</sup> Friske and Subbarow,<sup>19</sup> Kuttner and Cohen,<sup>20</sup> and Bodansky.<sup>21</sup> Since 1925 when it was

pointed out that the shortcomings of quinol as the phosphomolybdic acid-reducer could be avoided by using aminonaphtholsulfonic acid instead,<sup>19</sup> the latter has been the popular choice. Wainwright<sup>22</sup> adapted the stannous chloride method of Bodansky for use with whole saliva for his extensive studies in this field, and this method has been applied recently to parotid fluid by Chauncey and Weiss.<sup>23</sup>

The principal problem in salivary inorganic phosphate procedures is the difficulty in obtaining trichloroacetic acid filtrates that will remain clear following the addition of the ammonium molybdate reagent. This problem was pointed out by, among others, Davies and Rae,<sup>24</sup> who thus were forced to estimate inorganic phosphate indirectly by determining total phosphate after perchloric acid digestion of saliva.<sup>25</sup> Inorganic phosphate then was precipitated by adding magnesia mixture, and a second total phosphate determination was carried out on the filtrate. Their results were not in accord with those of Lura<sup>26</sup> and, after published exchanges of criticism it was concluded<sup>27</sup> that both methods were subject to considerable experimental error, but that no better method was available at that time.

Announcement of the AutoAnalyzer as a means of substituting dialysis for the chemical preparation of protein-free filtrates suggested the possibility of successful direct salivary inorganic phosphate determinations. After dialysis of a series of untreated parotid fluid samples in this machine, however, a considerable number of the samples still clouded when exposed to the molybdate reagent. Since the inorganic phosphate content of parotid saliva is usually more than twice that of blood serum, it was possible to dilute the samples with varying amounts and concentrations of protein precipitants in an effort to remove the factor(s) that would persist after dialysis and produce the cloudiness with molybdate. It was found that a 1:5 dilution of parotid fluid with 10 per cent trichloroacetic acid provided a sample with sufficient inorganic phosphate content for successful determination; furthermore, addition of molybdate to the dialyzate of this TCA-treated fluid invariably resulted in a perfectly clear solution. It thus appeared that at least two factors, one dialyzable and one nondialyzable, were responsible for the cloudiness, since both treatments were necessary to insure removal of the complicating factor.

For AutoAnalyzer inorganic phosphate determination,<sup>19</sup> 1.0 ml. parotid fluid is added slowly, with agitation, to 4.0 ml. 10 per cent TCA. After standing at room temperature for 15 min., the tubes are centrifuged at 2000 rpm for 5 min. and the supernatants removed for analysis. The TCA-treated sample is aspirated into the AutoAnalyzer manifold, mixed with air and physiological saline, and dialyzed against a stream of aminonaphtholsulfonic acid solution. Sulfuric-molybdate reagent then is mixed with the phosphate-ANSA solution, color is developed at 95° C. and read at 660 m $\mu$  in a 10-mm. flow cuvette. The 10-mm. light path provides the additional sensitivity needed with this diluted sample.

Samples may be processed at the rate of 40 per hour, but when there is a substantial difference in inorganic phosphate content of successive samples, sample-to-sample contamination is often noted; for example, if one sample is of very high phosphate concentration, the next sample reading will be higher than the true value. Operation at 40 samples per hour with air or water spac-

ing (20 parotid fluid samples per hour) is somewhat more satisfactory and considerably reduces this contaminating effect; however, since sample size is not a problem with this diluted specimen, we prefer the more reproducible and contamination-free method of 20 per hour without spacing. It was under the latter experimental conditions that the following data were collected.

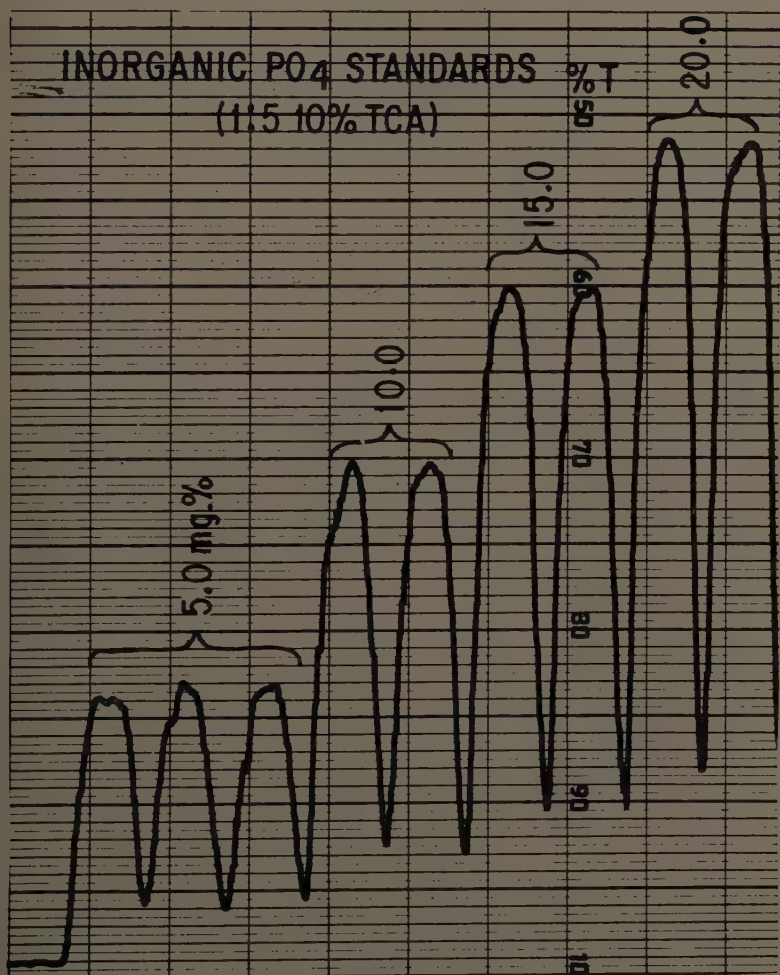


FIGURE 6

FIGURE 6 is a recorder tracing for TCA-diluted standards simulating 5.0, 10.0, 15.0, and 20.0 mg. per cent inorganic phosphate standards. Semilogarithmic recording, obtained by nonlinearizing the recorder, produces these relatively high peaks. Subsequently, to obtain a linear plot and to facilitate machine calculation, per cent transmission readings are converted to optical densities and calibration curves are tested by plotting on linear graph paper.

FIGURE 7 presents a typical standard curve of optical density versus concentration for the 5, 10, 15, and 20 mg. per cent simulated standards; also depicted is the accuracy with which added known amounts of phosphate are recovered by this procedure. To portions of a large pooled parotid fluid specimen, found to contain 12.55 mg. per cent inorganic phosphate, was added sufficient phosphate to produce the theoretical values of 14.30, 16.05, and 17.80 mg. per cent. A mean recovery of 99.8 per cent resulted.

Duplicate determinations were carried out on a series of 40 parotid fluid samples, and the coefficient of variation for the method was found to be 1.07 per cent. The mean inorganic phosphate value for all determinations was 11.38 mg. per cent, and the standard deviation was 0.122.

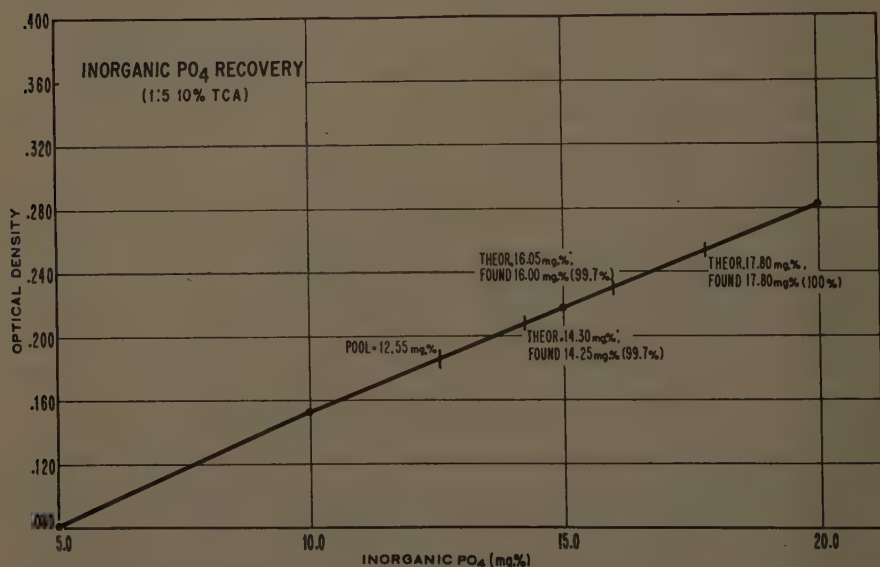


FIGURE 7

### *Urea Nitrogen in Parotid Fluid*

AutoAnalyzer determination of urea nitrogen is based upon the discovery by Fearon<sup>28</sup> in 1939 that color is produced by reaction of urea with diacetyl monoxime and oxidation of the product. Ormsby<sup>29</sup> applied the reaction to blood and urine and found that the results were more accurate than those then obtainable by other methods. He reported mean recovery rates of 100.3 per cent in urine and 100.6 per cent in blood. Rosenthal<sup>30</sup> checked the effect of acid concentration, diacetyl monoxime concentration, urea concentration, and heat on color production. He found that, although the reaction was not specific for urea, some color being produced by compounds containing similar groups, only urea gave a yellow color with an absorption maximum at 480 to 485  $\mu$ . Over the normal range, his values for blood urea agreed with those obtained by nesslerization methods but, at higher concentrations, the



diacetyl monoxime method gave values up to 8 per cent higher. Skeggs<sup>4</sup> pointed out that distinctly high results were obtained on blood samples read as less than 20 per cent transmittance, and that samples falling in this range should be diluted and retested. He compared the AutoAnalyzer method to the urease-aeration-titration procedure,<sup>31-33</sup> and found an average difference for 40 samples of 0.85 mg. N/100 ml. His values for the continuous method were corrected by a factor of 0.95, determined from his recovery experiments. Marsh *et al.*<sup>5</sup> evaluated the AutoAnalyzer by comparing it with a urease-direct nesslerization procedure<sup>34</sup> for blood urea nitrogen. Replicates on the AutoAnalyzer indicated a standard deviation of 1.3 mg. urea N/100 ml., much of this difference being due to interpretation of the recorded peaks. The standard deviation of the absolute difference between the results obtained by the two methods was 2.1 mg. per cent, the results obtained by the diacetyl method being slightly higher (S.D. = +0.17). Recovery with the urease method was relatively low (av. 94.5 per cent), suggesting that the reactions of the urease method were not completed under the conditions utilized by these investigators.

The concentration of urea nitrogen in parotid fluid is 76 to 90 per cent of that in blood,<sup>35-38</sup>; hence measurement on the AutoAnalyzer may be carried out by the recommended macro method. By this method samples may be processed at 20 or 40 per hour. Untreated parotid fluid is mixed with NaCl diluent and dialyzed against a stream of diacetyl monoxime. The urea-laden diacetyl monoxime then is combined with sulfuric-arsenic pentoxide reagent and the mixture is heated at 95° C. The color developed is read at 480 m $\mu$  in a 6-mm. flow cuvette. The results with parotid fluid compare well with those reported for blood.<sup>4,5</sup>

In order to conserve the sample, however, an attempt was made to adapt the established micro method to the analysis of the TCA-treated parotid fluid prepared previously for inorganic phosphate analysis. To balance the effect of TCA on sample readings, the urea nitrogen standards also were diluted 1:5 with a 10 per cent solution of this acid.

Neither standards nor samples can be processed successfully by this method at a rate exceeding 20 per hour. Variation occurs between duplicate measurements of 5.0 to 37.5 mg. per cent standards processed at 40 per hour. At this speed a low-concentration parotid fluid sample is affected by a preceding high-concentration sample. FIGURE 8 outlines color development for samples aspirated at 40 per hour, 20 per hour, and continuously until a full sample cup was drained. Color development was virtually complete at 20 per hour. After further testing it was found that the unspaced 20-per-hour procedure was the method of choice but that, if sample size was a consideration, air-spaced samples could be processed successfully at 40 per hour.

With the sulfuric-arsenic pentoxide reagent sensitized so that a 150-mg. per cent standard read about 20 per cent transmission, as recommended for the blood method, the micromethod standard curve linearity broke at 15.0 mg. per cent, the slope decreasing below this point. From tests with varying amounts of sulfuric acid in the reagent, it was found that optimal results were obtained when the sensitivity was such that a 37.5 mg. per cent TCA-treated

standard (actual parotid fluid concentration: 7.5 mg. per cent) had a transmittance of 54 to 55 per cent when processed through the micromanifold and read in the 10-mm. flow cell. With this sensitization and in the absence of sample-to-sample contamination, the plot of optical density against concen-

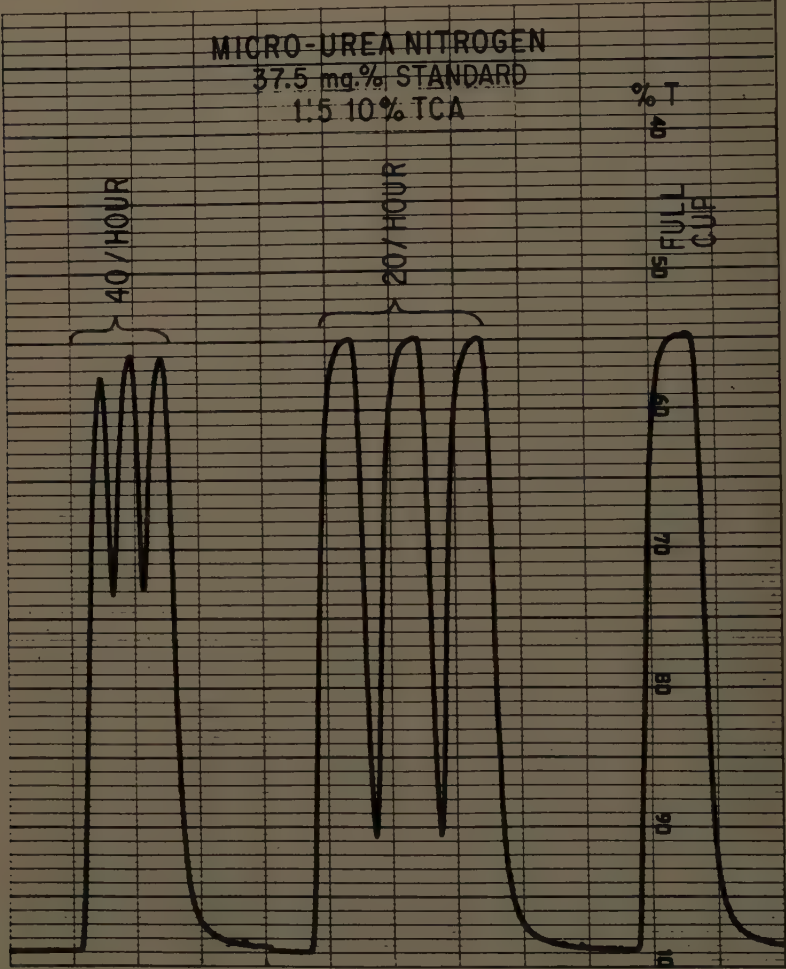


FIGURE 8

tration is linear. FIGURE 9 demonstrates the character of the peaks and the degree of reproducibility when duplicate parotid fluid samples are processed consecutively. Slight differences may be noted between duplicates for the higher-concentration specimens, the first sample usually giving a slightly lower reading than the second, but the lower-concentration samples are not affected by a preceding high-value specimen. For greater accuracy, high-concentration samples may be diluted further and reprocessed.

Duplicate analyses were carried out on 40 parotid fluid samples. The coefficient of variation for the method was found to be 1.12 per cent, the mean value for all samples being 11.77 mg. per cent and the standard deviation 0.132.

Recovery of added known amounts of urea nitrogen by this procedure is illustrated in FIGURE 10, which shows also a typical standard curve. A parotid pool value of 8.00 mg. per cent was increased to 9.84, 13.52, and 17.20 mg. per cent, and the recoveries were 100.6, 101.7, and 103.2 per cent, respectively.

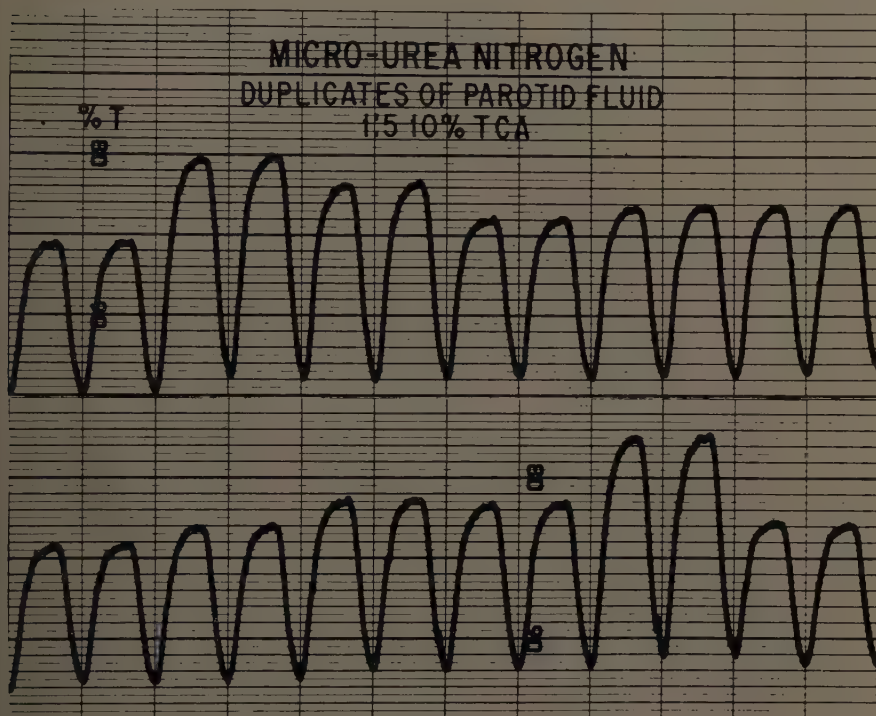


FIGURE 9

### *Salivary Glucose*

Whether glucose is present in saliva has long been a subject of research controversy. In view of the generally accepted chemicoparasitic concept of dental caries initiation<sup>39</sup>—that fermentable carbohydrates are converted to acid by oral microorganisms and that this acid, in turn, decalcifies tooth structure—the importance of the amount of glucose present in saliva is obvious. It is also of interest to determine whether a reported lack or relative lack of glucose in saliva is due to a threshold mechanism in the gland cells, or whether the metabolism of glucose in the glands might be held responsible.

In 1891, Weyert<sup>40</sup> reported that there was no glucose in the saliva of a normal dog, but that if the blood sugar level were raised to very high levels, glucose

was then present in the saliva. These results were confirmed later by Asher<sup>41</sup> and by Hebb and Stavraký.<sup>42</sup> The latter investigators administered adrenaline and found glucose in the saliva even when the blood glucose level was low; this led to the conclusion that the drug had altered the permeability of the gland cells. Langley *et al.*<sup>43</sup> found in dog studies that the parotid gland threshold for glucose was  $512 \pm 51.5$  mg. per cent, that intravenous insulin raised this threshold to 1200 mg. per cent, and that, in alloxan-diabetic dogs, glucose was present in parotid saliva at a fasting blood glucose level of 269 mg. per cent. Amberson

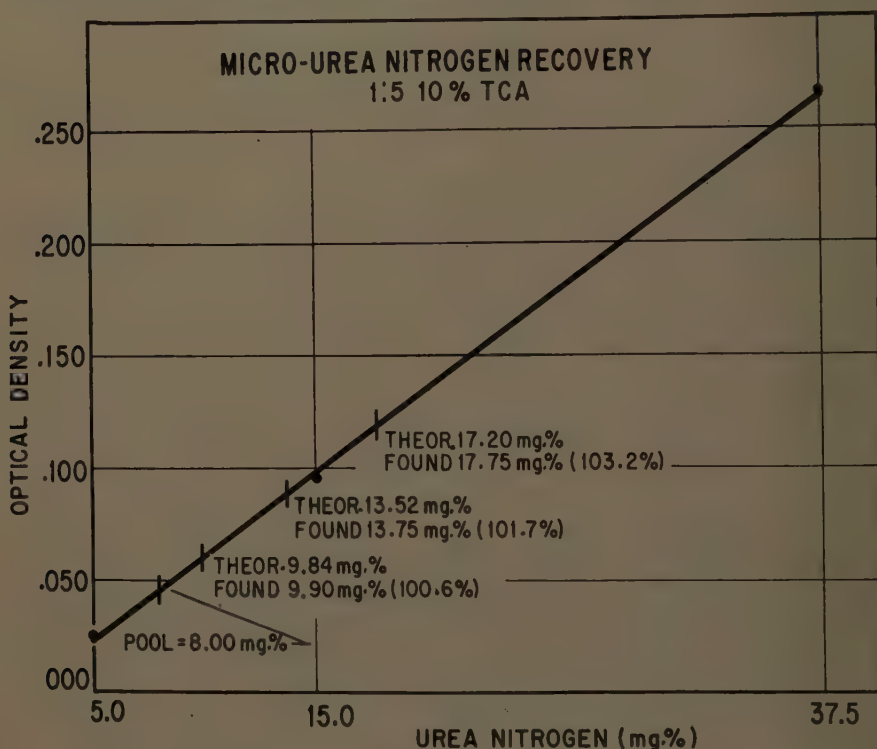


FIGURE 10

and Hober<sup>44</sup> studied the isolated submaxillary gland of the cat and concluded that the gland was permeable only slightly or not at all to glucose. Updegraff and Lewis<sup>37</sup> could not find a measurable amount of glucose in 68 human saliva specimens; furthermore, they determined that the administration of 300 gm. glucose did not increase the saliva levels. Chauncey\* was not able to find glucose in the parotid fluid of normal humans undergoing glucose tolerance tests. Berggard and Werner<sup>45</sup> state that it is well known that saliva does not contain appreciable amounts of free polysaccharides or sugars, but in a pooled specimen they found traces of glucose by paper-partition chromatography.

\* H. H. Chauncey, Tufts University School of Dental Medicine, Medford, Mass. Personal communication, 1959.



A Japanese group<sup>46</sup> found the sugar content of sweat to vary from 1.3 to 10.5 mg. per cent, but concluded that there was no sugar in normal saliva. They found measurable quantities, however, in the saliva of diabetics and in normal subjects given glucose.

With the AutoAnalyzer, glucose may be determined in parotid fluid with no prior treatment of samples and with no modification of the recommended micro-method for whole blood, serum, plasma, and cerebrospinal fluid. The color loss resulting from the reduction of the yellow solution of potassium ferricyanide to potassium ferrocyanide is read at 420  $m\mu$  in a 3-mm. flow cuvette. This characteristic loss of color was described by Hagedorn and Jensen in 1923,<sup>47</sup> and the photoelectric measurement of this color diminution was utilized in the determination of glucose in blood and urine by Hoffman in 1937.<sup>48</sup>

The determination of glucose in saliva should not be performed at a rate exceeding 20 glucose samples per hour. When 40 samples are run each hour with no spacing, contamination is present; while at 60 per hour with air spacing, contamination is not evident. Color development at this speed is less, however, than at the rate of 20 per hour or 40 per hour, the latter with air or water spacing. Since it was felt that the 20-per-hour unspaced peaks were too broad for accurate reading, and since water spacing seemed to offer no advantages over air, the 40-per-hour method with air spacing was taken as the method of choice. Another reason for avoiding the 20-per-hour nonspaced procedure was that it requires a virtually prohibitive 1.6 ml. of undiluted specimen. The method was tested against prolonged aspiration of standards, and it was established that maximal color production was gained by this approach.

The standard glucose transmission curve extending from 0.4 to 3.0 mg. per cent glucose, determined at 40 samples per hour with air spacing, is linear. When this curve is extended upward to include the 4.0 and 5.0 mg. per cent standards, a definite break is noted at the point of the 3.0 mg. per cent standard. The response of the linearized recorder from 3.0 to 5.0 mg. per cent is linear within itself, but the slope of the line differs slightly from that found between 0.4 and 3.0 mg. per cent. This offers no particular difficulty in parotid fluid analysis, since it is most uncommon that a glucose concentration, even during a tolerance test, exceeds 3.0 mg. per cent. Neither is difficulty experienced at the lower end of the curve, since virtually all samples will contain at least 0.4 mg. per cent glucose.

FIGURE 11 demonstrates the general configuration of the recorder response when 1.0, 2.0, and 3.0 mg. per cent standards (at the right of the graph) precede a parotid pool glucose determination and three glucose recovery analyses. For exactness in measuring the first sample following the highest standard, a waste sample of the parotid fluid was included. The reading for this sample is higher than that for the replicates directly following it. It is a routine practice in our laboratories to follow standard runs with at least one sample of the fluid to be analyzed.

FIGURE 12 outlines the recovery by this method of known amounts of glucose added to portions of a parotid fluid pool. The pool glucose value was 1.10 mg. per cent; after it was elevated to 1.48, 1.86, and 2.23 mg. per cent by these additions, recoveries of 98.0, 102.2, and 98.7 per cent were found.

Forty parotid fluid samples were analyzed in duplicate and the coefficient of variation for the method was found to be 5.26 per cent, the mean for all 40 analyses being 1.14 mg. per cent and the standard deviation, 0.060.

Whether or not this reducing substance is true, glucose is not definitely established by the above procedures. As a further check on the method, two

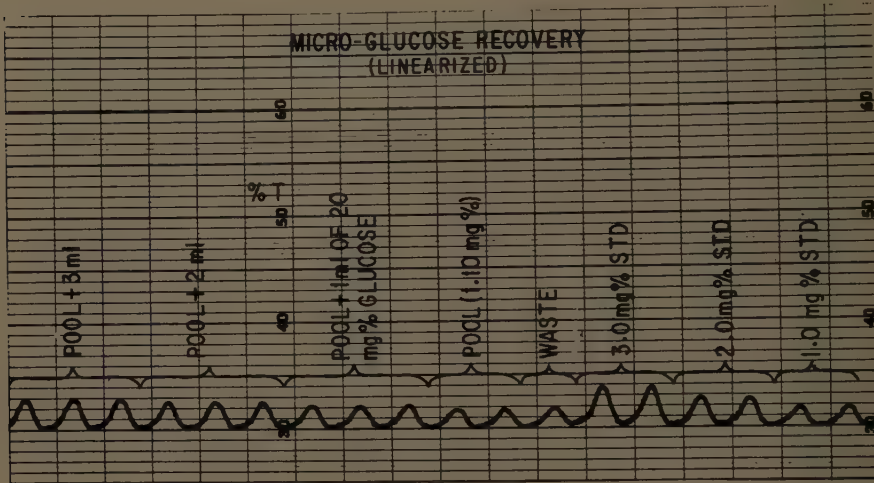


FIGURE 11

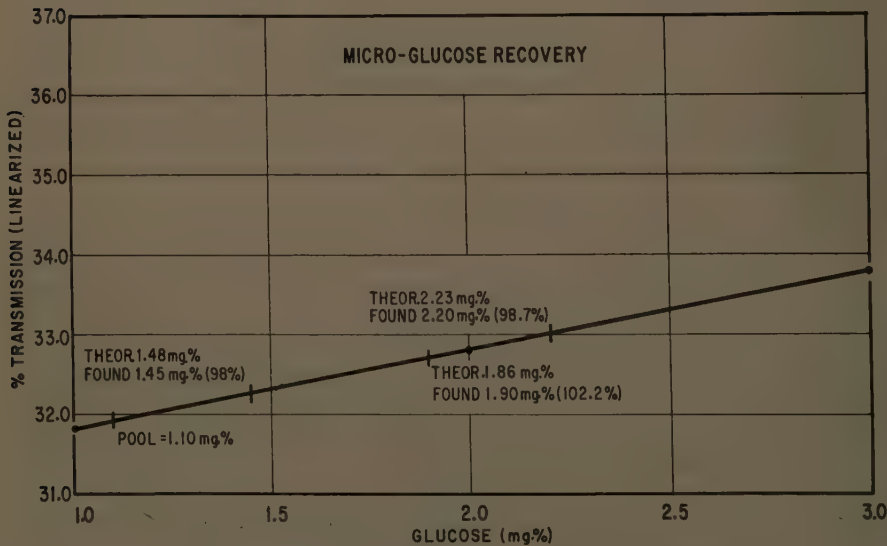


FIGURE 12

vials of Versatol-A\* abnormal blood chemistry control were prepared and analyzed for glucose. The control was processed in quintuplicate and read consistently between 2.0 and 2.1 mg. per cent. Our 1:100 dilution had been made from a 205 mg. per cent control. In the light of these and other findings we are accepting these AutoAnalyzer determinations as true glucose.

### Summary

Automatic methods of chemical analysis have been adapted to parotid fluid analysis and evaluated in determinations of calcium, inorganic phosphate, urea nitrogen, and glucose. TABLE 1 presents means, standard deviations, and coefficients of variation for duplicate analyses by these methods. Inorganic phosphate and urea nitrogen were determined on a 1:5 dilution of parotid fluid in 10 per cent TCA. Dialysis of this prepared sample in the AutoAnalyzer corrected completely the heretofore inevitable complication, inherent in the phosphate method, of turbidity development upon addition of molybdate. This 1:5 diluted sample contained sufficient urea nitrogen for accurate measure-

TABLE 1  
AUTOANALYZER METHODS FOR PAROTID FLUID

Variable*	Mean	Standard deviation	Coeff. of variation %
Inorganic phosphate	11.38 mg. %	0.122	1.07
Calcium	1.53 mEq./l.	0.066	4.31
Urea N	11.77 mg. %	0.132	1.12
Glucose	1.14 mg. %	0.060	5.26

\* Forty sets of duplicates analyzed for each variable.

ment by the micro procedure. For calcium, the method was modified in that the standards were prepared in 0.4 M NaCl and the Hyamine solution was made to contain 0.03 M NaCl. A linear glucose response in the desired range was elicited by the use of an undiluted sample whose color loss was read in a 3-mm. flow cuvette. In contrast to results given in the existing literature, a measurable level of reducing substance, accepted as glucose, was found in all samples.

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## AUTOMATIC ANALYSIS OF AMINO ACIDS\*

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Interest in amino acids is directed most often toward considerations of the contents or proportions of specific amino acids in a mixture. In order to determine the amino acid composition of a protein, it is necessary first to hydrolyze the protein and free the constituent amino acids from peptide linkage. From this hydrolysis results a complex mixture. In biological materials, free amino acids as well are almost invariably found as mixtures. Thus the most essential and critical requirement for a general automatic chemical method of analysis is a continuous system for automatic separation of individual amino acids from a mixture prior to detection. This can be accomplished efficiently now by ion-exchange chromatography.

In 1944 Elsdon and Synge<sup>1</sup> demonstrated that amino acids could be separated by partition column chromatography with the use of raw potato starch as a supporting medium for an aqueous phase over which sample and organic solvents were passed. In experiments with hydrolysates of gramicidin, Synge<sup>2</sup> used *n*-butanol-water as the solvent system and collected the effluent in a series of relatively large fractions, each of which was tested qualitatively with ninhydrin paper.

In 1948 Moore and Stein described an adaptation of the starch column to quantitative procedures.<sup>3-5</sup> The effluent, rather than being collected from the column in large fractions, was divided into relatively small fractions of precisely measured volume. For distributing the fractions into tubes, Stein and Moore developed the automatic fraction collector.<sup>4</sup> The automatically collected fractions were analyzed by a quantitative colorimetric ninhydrin procedure.<sup>6</sup> Starch columns had low capacities, and resolution was found to be disturbed by the presence of metals. Fluids of high salt content, such as blood plasma or urine, required desalting prior to chromatography, and the effluent contained traces of carbohydrate which interfered with the isolation of pure compounds. On starch columns some of the amino acids did not travel at rates compatible with their distribution coefficients; this indicated that starch, the inert supporting medium for the aqueous phase, also possessed weak ion-exchange properties.

In 1951 Stein and Moore<sup>7</sup> substituted finely divided sulfonated polystyrene, a commercial ion-exchange resin, for starch in chromatographic columns. They used aqueous buffers in an elution system that separated all of the amino acids found in protein hydrolysates. Thereafter, modifications and refinements in ion-exchange chromatographic techniques and methods for quantitative detection of amino acids and related compounds with ninhydrin reagent were reported from several laboratories in a number of publications.<sup>8,9</sup>

There was still a need to increase the speed and efficiency with which these

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analyses could be performed. In 1954 Darrel H. Spackman, in association with Stein and Moore, undertook to develop equipment that would automatically process column effluent and record the results, to obviate dependency on involved manual operations.

The approach that was followed forms the basis for presently available commercial equipment, the design for which is shown schematically in FIGURE 1.<sup>10,11</sup> The principal component of the system is the ion-exchange column. The sample is introduced at the top of the column, where a line is connected to a pump that delivers buffer to the column at a constant volume and forces buffer and sample through the resin bed. The buffer should be divested of dissolved

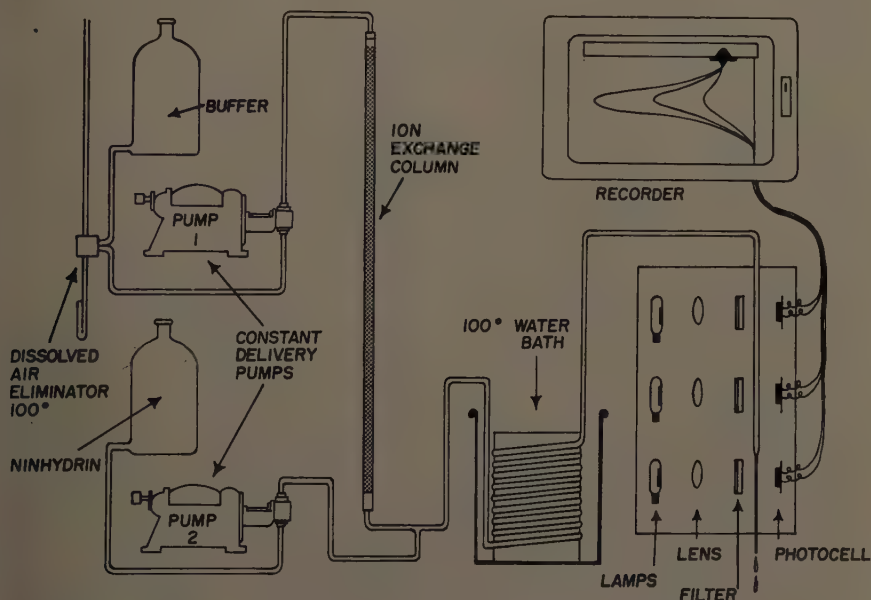


FIGURE 1. Schematic diagram illustrating design of Spackman-Moore-Stein apparatus for amino acid analysis.

air before entering the pump in order to maintain accurate delivery volumes and to preclude dissipation of bubbles in the column, which would lead to channeling. Since each of the amino acids has a different coefficient of distribution between the flowing aqueous phase and the stationary resin phase, the acids become separated during passage through the resin bed. They are then eluted in a predictable sequence from the column, and the effluent joins a stream of ninhydrin reagent delivered from a reservoir by a second constant-volume pump. For optimal reactions of the ninhydrin reagent with the amino acids, a heating time of 15 min. at 100° C. is provided by passage of the stream through a capillary coil of appropriate length immersed in a boiling water bath. The blue and yellow colors indicating the presence of amino acids are detected during subsequent passage through a photometer consisting of three units.

Three curves of absorbance values, at 440  $m\mu$  and for two solution depths at 570  $m\mu$ , then are plotted against time by a multipoint recorder.

Before amino acid analyzers were available commercially we designed and constructed an instrument employing the principles and equipment described by Spackman *et al.* for use in our studies of the pathological proteins derived from patients suffering from multiple myeloma.<sup>12</sup>

The general design of our instrument was adopted subsequently for production of a version now commercially available.\* Our general design criteria were (1) that the apparatus should follow the plans of the original authors with the greatest possible fidelity, (2) that the apparatus should be enclosed in a movable unit, and (3) that the enclosure should not inhibit access to or inspection of any part or unit of the instrument. These criteria were fulfilled with complete satisfaction by installing the equipment on shelves, panels, and aluminum support rods attached to commercially available transmitter racks,† as seen in FIGURE 2. Access to the interior of these cabinets may be achieved from top-opening doors, removable side panels, and full-length doors, both rear and right front. In addition to easy inspection, adjustment, and maintenance, this arrangement has permitted easy rearrangement of existing equipment or rapid replacement of certain component parts as improved units and accessories have been developed. (The shorter unit on the left in FIGURE 2 is a General Measurements scintillation counter‡ recently appended to our unit for radioactive carbon measurements.) We have used the instrument chiefly in studying amino acid compositions of proteins related to gamma globulin, and it is interesting to compare the efficiency of automation with the tedium of previous methods in this case. A complete amino acid analysis of a protein hydrolysate can now be obtained within 24 hours. Just 15 years ago, the first analysis of the complete amino acid composition of a single protein preparation,  $\beta$  lactoglobulin, performed by a single group of workers was reported by Brand *et al.*<sup>13</sup> In 1946, Brand reported the amino acid compositions of some additional proteins as determined by 39 different microbiological and chemical procedures.<sup>14</sup> One of the proteins was gamma globulin, which we have analyzed by the new automatic method. Our results are compared with those reported by Brand in TABLE 1. Considering that the two samples were from different sources and that the methods of analysis are unrelated, the results are remarkably similar. Whereas until a decade ago weeks of arduous labor were required to determine amino acid compositions, now in a rapidly increasing number of laboratories such analyses are made daily, by virtue of the new automatic equipment.

Although the new equipment has greatly facilitated analysis, the questionable degree of correspondence between amino acid composition of intact proteins and their respective acid hydrolysates continues to challenge the authenticity of results obtained by present methods. An obvious problem is encountered in the destruction of tryptophan, and considerable losses of hydroxy-amino acids are also evident. Available data in the literature indicate that

\* Model K-5000 Amino Acid Analyzer; Phoenix Precision Instrument Co., Philadelphia, Pa.

† 1-PR-70-24, 1-PR-70-24 less front door, 2-SP-70-24 with louvers; Premier Metal Products Co., New York, N. Y.

‡ Model 3000; General Measurements Div., Precision Scientific Co., Garnerville, N. Y.



such losses vary with the type of protein involved. Recent work by Hill<sup>16</sup> offers encouraging support to the idea that complete enzymatic hydrolysis preceded by mild denaturation may yield better results for some proteins.

Blood plasma, urine, and other physiological fluids can be analyzed rapidly with automatic equipment to yield information on as many as 50 ninhydrin-reactive compounds. A high-pressure ultrafiltration apparatus suggested by

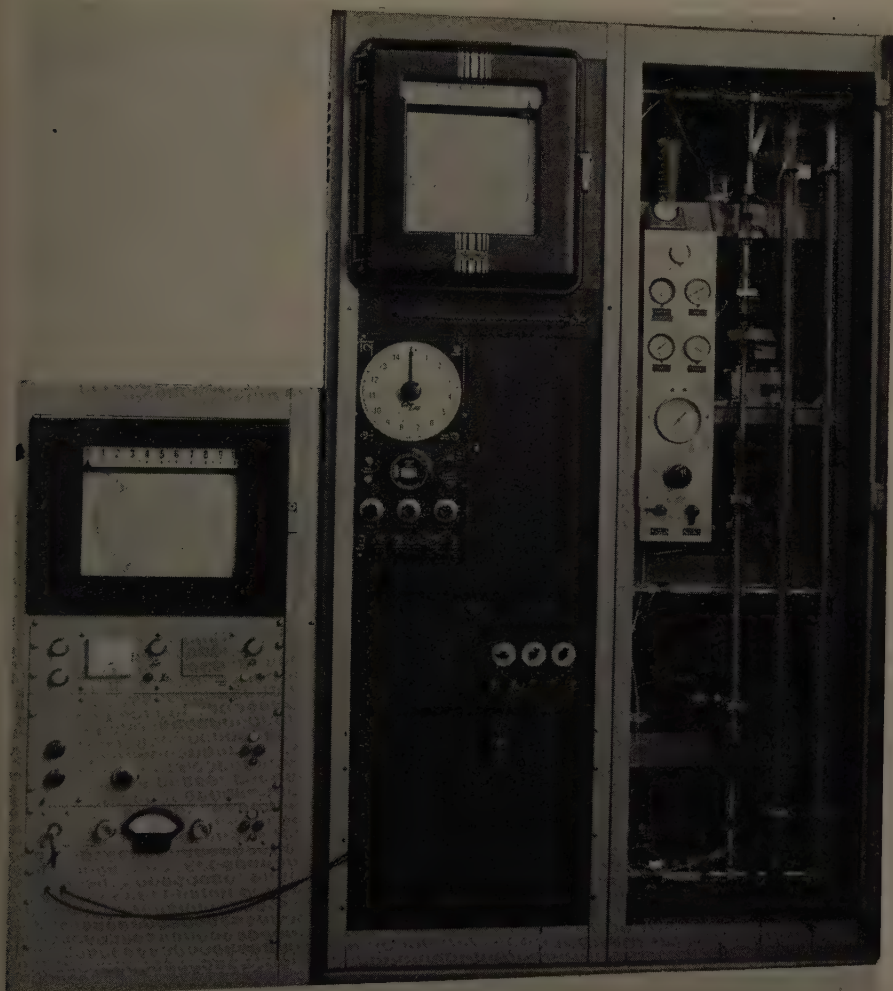


FIGURE 2. A front view of the amino acid analyzer as now used in the laboratory. The components and reagents described by Spackman, Stein, and Moore are all housed compactly inside a commercially available enclosure. The shorter unit at the left is a General Measurements Div., Precision Scientific Co. Continuous Radioactive Liquid Scintillation Analyzer that employs a serpentine of plastic phosphor tubing optically coupled to a photomultiplier tube and standard electronic circuits. The efficiency in counting  $C^{14}$  is approximately 10 per cent.

Rosen and Levenson<sup>17</sup> has been found useful for rapid preparation of plasma for amino acid analysis.\*

The new automatic instrumentation thus far has been used primarily for direct analytical work. In the Spackman-Stein-Moore system, detection of amino acids separated by ion exchange is dependent on the addition of a color-producing reagent with conversion of amino acids to other products. In many instances it is desirable to isolate compounds by ion-exchange chromatography for further testing and evaluation. The analytical system cannot be scaled

TABLE 1  
AMINO ACID COMPOSITIONS OF HUMAN GAMMA GLOBULIN

Amino acid	Cohn Fraction II (Grams amino acid residue per 100 gm. of protein)		
	Microbiological-chemical analyses (Brand, 1946)	Automatic chromatographic analysis (Squibb Fraction II)	
		20-hr. hydrolysate	70-hr. hydrolysate
Lysine	7.1	6.97	7.46
Histidine	2.21	1.76	2.44
Arginine	4.30	4.00	4.40
Aspartic acid	7.6	8.23	7.90
Threonine	7.1	7.34	6.70
Serine	9.5	9.49	7.96
Glutaminic acid	13.4	11.0	10.80
Proline	6.8	6.41	6.37
Glycine	3.2	3.66	3.46
Alanine	—	3.56	3.31
Half-cystine	2.07	2.06	1.88
Valine	8.2	7.98	8.14
Methionine	0.93	0.88	0.96
Isoleucine	2.3	2.14	2.15
Leucine	8.0	7.60	7.36
Tyrosine	6.08	5.88	5.72
Phenylalanine	4.1	4.49	4.24
Tryptophan	2.61	2.58*	—
Amide NH <sub>3</sub> †	1.35	1.61	2.04

\* Method of Goodwin and Morton.<sup>15</sup>

† Values not included in totals.

directly to preparative work, since the desired compounds would be destroyed by the colorimetric method of detection. Instead, it is necessary to divert a small fraction of effluent from a preparative column into the analyzer, while the bulk flows onward to a fraction collector. It is possible to use a peristaltic pump to effect a side stream from the main column effluent and, if the tubing employed has sufficiently small internal diameter, resolution will not be compromised appreciably. Pumps of this type have some disadvantages, however. They do not maintain precise volume delivery over extended periods, and they cannot be used to pump against more than slight resistances. Nevertheless, they are capable of very low volume-delivery rates, and high resistances are not encountered in the present application. In most instances the volume

\* ARAflo Ultrafiltration Apparatus; Applied Research Associates, New York, N. Y.

delivery accuracy required for monitoring a preparative column would be less than that for analyzing protein hydrolysates, and when the peristaltic pump is used in conjunction with specially formulated vinyl tubing, satisfactory results can be obtained by diverting as little as 1 ml./hour from the main stream. FIGURE 3 presents a schematic diagram of a system that has been employed successfully in our laboratory for combined analytical-preparative analysis.

Buffer is delivered to the column at  $V'_{ef} = 47.2$  ml./hour/cm.<sup>2</sup> of superficial column cross section. This corresponds to 30 ml./hour for an 0.9-cm. column, the size of the standard analytical column supplied with Phoenix and Spinco instruments. Maintenance of  $V'_{ef}$  at this volume with larger columns results

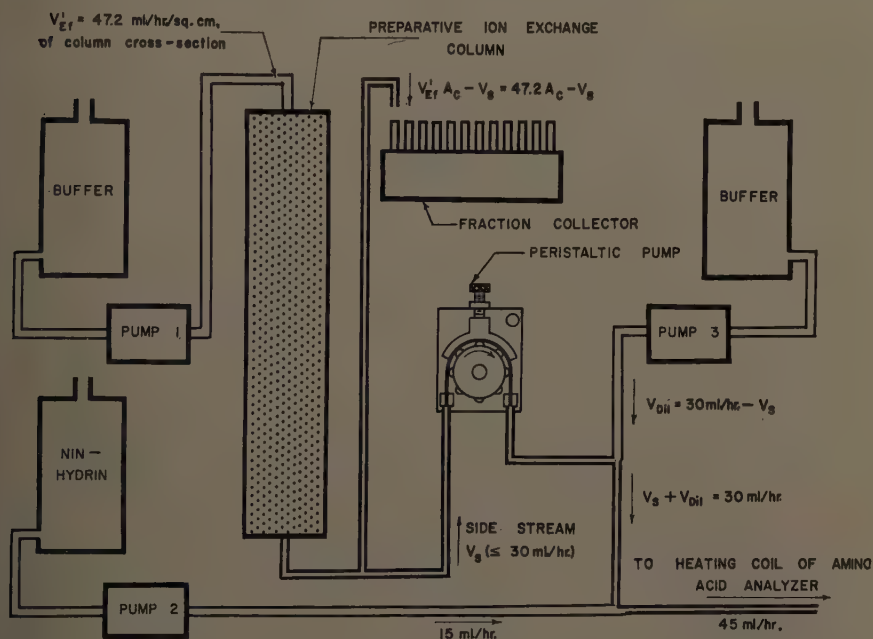


FIGURE 3. A system for analytical-preparative ion-exchange chromatography of amino acids

in similar linear displacement rates. The column diameter may therefore range from 0.9 cm. up to a size for which  $V'_{ef}$  equals the capacity of the pump. A small fraction  $V_s$  of the effluent emerging from the column is metered into a side stream, and the remainder  $V'_{ef} A_c - V_s$ , where  $A_c$  is the column superficial cross-sectional area, overflows into a volumetric fraction collector. The relay that advances the fraction collector also sends a pulse to an indexing pen on the recorder so that each tube collected is registered by a pip along the base line of the recorded chromatogram. The side stream is created by the operation of a 1- or a 6-rpm peristaltic pump,\* employing 0.018- by 0.046-inch vinyl tubing threaded through  $\frac{1}{16}$ - by  $\frac{1}{18}$ -inch vinyl tubing.† To the side stream is added

\* Such as that made by the New Brunswick Scientific Company, New Brunswick, N. J.

† Phoenix Precision Instrument Company, Philadelphia, Pa.

buffer from the alternate column pump, at  $V_{dil} = 30$  ml./hour  $- V_s$ , bringing the total volume again to 30 ml./hour and re-establishing a stream volume equivalent to the effluent flow rate employed in the standard analytical procedure. Ninhydrin reagent at a volume of 15 ml./hour is added, and the stream flows onward into the heating coil and photometer of the analyzer. Since the essential volumes, rates, and column displacement velocities have been adjusted at critical points to simulate the standard analytical procedure, the time required for development of the chromatogram as well as its shape and form will remain unchanged. It has also been found that color yields are not significantly different.

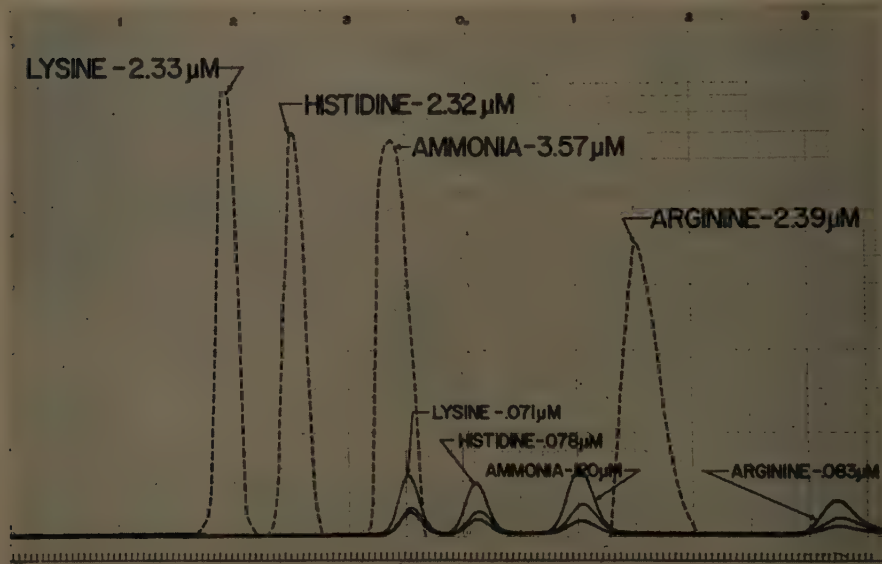


FIGURE 4. Recording and plot of combined preparation and analysis of basic amino acids.

FIGURE 4 shows results of an analysis made according to this scheme, with the use of the standard 0.9- by 15-cm. column. Approximately  $\frac{1}{30}$  of the effluent was directed into the side stream, diluted up to 30 ml./hour, and analyzed, while the bulk of the material was distributed into tubes by a fraction collector. One-ml. fractions were collected and analyzed manually according to the method of Moore and Stein.<sup>18</sup> The results were plotted on a strip chart that also recorded the automatic analysis of the side stream. The tube-indexing marks at the bottom of the chart represent 1-ml. fractions and serve as the abscissa for the dashed curves. The time lag between the appearance of an amino acid in the fraction collector and the registration of the acid in the side stream to the analyzer is due to the time required for passage from the column to the peristaltic pump and then to the manifold and through the heating coil to the analyzer. It is desirable to keep tubing lengths for these connections



at a minimum. The total time lag must be determined in order to establish the correspondence between the analyzer peak and the tube filled.

The quantitative aspects of this experiment are summarized in TABLE 2. Recovery of the amino acids is quite satisfactory, and the predicted proportionation correlates well with the measured flow rates. In instances where relatively small amounts of a compound are to be encountered, or where the ninhydrin color yield might be small (for example, in the case of peptides), it would be prudent to take a larger fraction of effluent for analysis. We have used a 6-rpm pump with  $\frac{1}{64}$ -inch i.d. tubing to obtain  $V_s$  values of approximately 6 ml./hour; under these conditions, the analytical system operates at  $\frac{1}{5}$  rather than  $\frac{1}{30}$  of the sensitivity of the standard Spackman-Stein-Moore analytical method. A 1-rpm pump equipped with  $\frac{1}{32}$ - or  $\frac{3}{64}$ -inch. i.d. tubing would accomplish the same result. A graduated selection of small-bore, properly formulated vinyl tubing is available,\* and those anticipating this type of operation are now advised to use the 1-rpm pump.

TABLE 2

RESULTS OF ANALYTICAL-PREPARATIVE CHROMATOGRAPHY OF BASIC AMINO ACIDS  
(Peristaltic pump, 1 rpm; 0.018  $\times$  0.046-in. vinyl tubing; 15  $\times$  0.9-cm. S.P.S. column;  
 $V_s$  = 1.1 ml./hr. to analyzer; overflow, 28.9 ml./hr. to fraction collector.)

Amino acid	Amount in mixture ( $\mu$ moles)	Amount determined ( $\mu$ moles)			Recovery (%)
		Analyzer	Fraction collector	Total	
Lysine	2.50	0.071	2.33	2.40	96.0
Histidine	2.50	0.078	2.32	2.40	96.0
NH <sub>3</sub>	3.66	0.120	3.57	3.69	100.8
Arginine	2.50	0.083	2.39	2.47	98.8

Some of the modifications that can be made in operation of the recording equipment to permit work on a preparative scale or at faster elution rates can be derived from FIGURE 5. The abscissa gives column diameters in centimeters, and the ordinate gives pump delivery volumes in milliliters per hour. The standard analytical method is represented by a point corresponding to a volume of 30 ml./hour delivered to a column 0.9 cm. in diameter, resulting in a displacement volume of 47.2 ml./hour/cm.<sup>2</sup> of superficial column cross-sectional area. The 47.2-ml. line should be referred to in determining the delivery volume and column diameter required for the same displacement volume and hence the same elution rate in a preparative system.

In standard commercial amino acid analyzers, chromatographic pumps with adjustable delivery volumes of up to 320 ml. are available. With this type of pump, the standard elution rate is maintained in preparative columns up to 3 cm. in diameter. A 3-cm. column could carry 10 times the sample load carried by an 0.9-cm. analytical column. For higher loads, larger capacity pumps and columns of greater diameters can be used.

\* Phoenix Precision Instrument Company, Philadelphia, Pa.

In experiments aimed at increasing the elution rate  $V'_{ef} A_c$ , the standard ratio of effluent to reagent volumes, the 15-min. passage through the heating coil, and a pump delivery volume of 30 ml./hour could be maintained with a considerable increase in displacement rates by use of columns smaller than 0.9

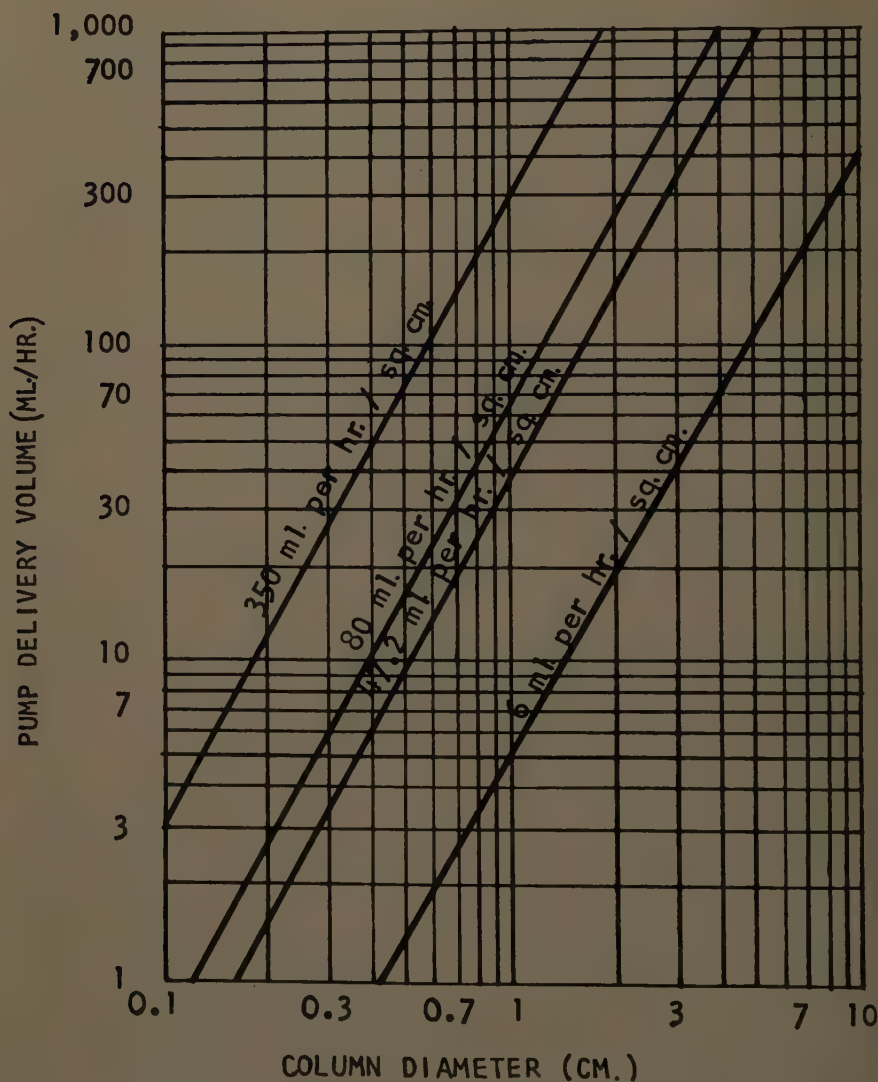


FIGURE 5. Flow rate conversions.

cm. in diameter. Flow resistance effected by the resin bed has been found to increase in direct proportion to the displacement volume, or volume per hour per square centimeter, up to displacements volumes of at least 350 ml./hour/

cm.<sup>2</sup> The volume for  $V'$  above which pressures begin to increase disproportionately has not been determined and would be dependent on the particle size distribution of the resin to be used. If a flow of 30 ml./hour through a 150-cm. length of resin in a 0.9 cm. tube results in a pressure of 47 psi, the pressure will be increased to 80 psi when the same volume is driven through an equal length of resin contained in a 0.7-cm. column, or to 235 psi in a 0.4-cm. column.  $V'$  in the first instance is 47.2 ml./hour/cm.<sup>2</sup>, in the second it is 80 ml./hour/cm.<sup>2</sup> and in the third it is 235 ml./hour/cm.<sup>2</sup>

Hamilton<sup>19</sup> has reported that chromatographic tubes as small as 0.4 cm. and displacement volumes up to 350 ml./hour/cm.<sup>2</sup> can be used with specially prepared ion-exchange resins to produce acceptable chromatographic resolution. With the present equipment it would seem feasible, on the basis of Hamilton's work, to operate at up to 235 ml./hour/cm.<sup>2</sup> using 0.4-cm. columns packed with smaller-sized ion-exchange resin particles, and a 30-ml./hour delivery volume. Maintenance of the standard 30-ml./hour volume would permit the usual passage time through the heating coil, thereby producing the usual color yields. These conditions would impose pressures of between 300 and 400 psi in the feed line to the 150-cm. column, but would reduce the running time from 16 $\frac{2}{3}$  to 3 $\frac{1}{2}$  hours. For the chromatogram to appear like the standard, the recorder chart speed would need to be increased accordingly. These considerations have been based on the presently used column lengths, ion-exchange resin, and elution schemes; undoubtedly, a variety of other systems can be devised for use with the automatic equipment.

Piez<sup>20</sup> has used 100- by 0.9-cm. columns of Dowex 50-by-12 for separation and elution of all of the amino acids found in protein hydrolyzates.<sup>20</sup> In his system a separate short column for rapid elution of the basic amino acids is not required, and the complete analysis can be performed with the use of automatic recording equipment within 24 hours. The resin, consisting of spherical particles 20 to 30  $\mu$  in diameter, contains 12 per cent divinylbenzene crosslinkage and is much less subject to swelling and shrinkage with changes in ionic environment than are the less cross-linked, sulfonated polystyrene particles. This property permits the use of gradient elution, and the multichamber varigrad device of Peterson and Sober<sup>21</sup> has been used to establish the gradient. The Piez system is relatively economical, a single column, two pumps, a varigrad, heating coil, photometer, and multipoint recorder constituting the minimum required equipment. Recently we have added a flow scintillation counter\* (see FIGURE 2) to our equipment; this device has now reached a stage of development that promises to make radioactivity measurements in conjunction with colorimetric determination of amino acids practical.

From these considerations, it seems feasible to increase the speed and versatility of the recording equipment, but there are many technical difficulties, particularly those involving the development of improved ion-exchange resins, to be encountered and overcome before this goal will be realized.

\* General Measurements Div., Precision Scientific Co., Garnerville, N. Y.

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# THE SOLUTION OF PROBLEMS INVOLVING SPECIAL HANDLING OF SAMPLES AND REAGENTS IN AN AUTOMATIC SYSTEM

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## *Introduction*

The impact of automation in analytical chemistry in the past few years has been and is being felt in nearly every field of science. The development of new equipment for faster and more accurate determinations permits more data to be collected, thus freeing investigators to carry on more basic research projects. In the Indianapolis laboratories of Eli Lilly and Co., the application of modern instrumentation to routine analytical problems led to automation with the use of the AutoAnalyzer.\* However, a new piece of equipment always invites speculation on techniques for extending its range of application. In attempts to expand the potential of any instrument, many problems are encountered. Consideration of a few of those problems and their possible solutions is the intent of this presentation.

## *Experimental Results*

The restrictions placed upon the AutoAnalyzer by the use of Tygon tubing in the manifold immediately concerned us because some of our potential applications required the use of organic solvents. A variety of different kinds of tubing was investigated for pumping these organics. None of the tubing examined possessed the characteristics necessary for the peristaltic pumping action, the heart of the entire system. If the tube did not crack, it stretched too tight to permit passage of the liquids. Parenthetically, I might add that subsequently we tried Silastic tubing,† and found that it does possess some of the necessary characteristics. However, we have not yet been able to install shoulders on this material to hold it in place on the manifold.

In order to circumvent the problem of tubing instability, a displacement technique was conceived. The theory was that if we could pump a solvent that was inert in the presence of Tygon into a container of the organic solvent, an equal volume of the organic could be displaced into a glass or polyethylene system. A polyethylene membrane in the form of a bag separates the two liquids. The organic reactive reagent is gravity-fed into the polyethylene bag, and the area outside of the bag is filled with HOH or some other inert solvent. The selection of the inert solvent likewise must be made so that no reaction would occur with the reagent in the bag in the event that the polyethylene membrane should break.

FIGURE 1 shows the latest version of our displacement container, disassembled. A two-gallon amber glass bottle was chosen as a container for the system.

\* Technicon Instruments Corp., Chauncey, N. Y.

† Technicon Controls Corp., Chauncey, N. Y.

A Teflon plug was fitted to the mouth of the bottle, and a brass pressure plate was constructed to produce an airtight seal. A thin hard-rubber ring must be placed between the bottle and its plug. Three glass tubes with Teflon stopcocks were tightly fitted into the plug. One of these serves as an inlet for inert solvent; the second, as an outlet for inert solvent and air; and the third, attached to the polyethylene bag by means of a Teflon gland, serves as the reagent inlet and outlet and therefore has two stopcocks attached to it. The polyethylene bag is fitted with a piece of polyethylene tubing that extends nearly to the bottom of the bag. This tube must be sealed thermally into the mouth of the bag with a brass block fitted to a pair of tongs. It is necessary to separate

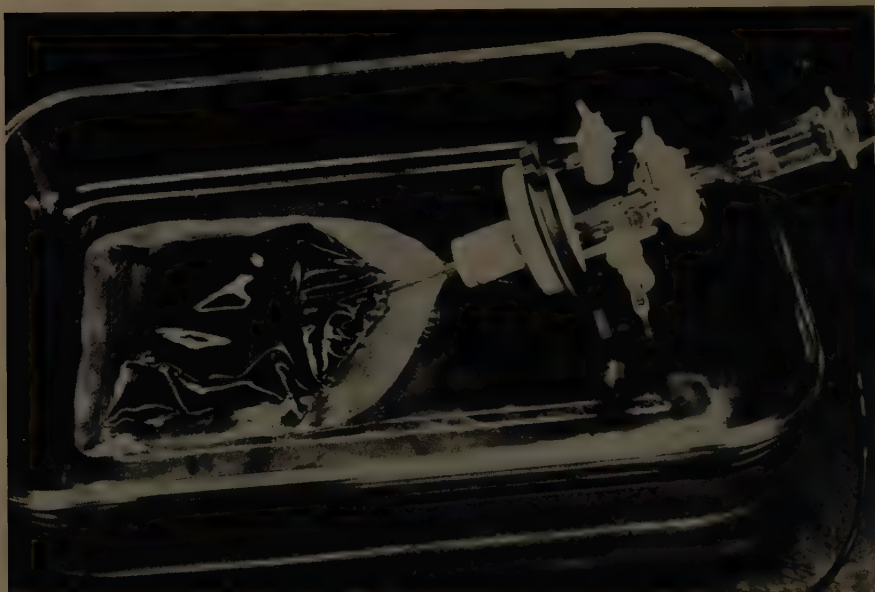


FIGURE 1

the sealing unit and the polyethylene with a sheet of waxed paper. The remainder of the bag is sealed with a soldering gun equipped with a flat tip. Again, the waxed paper must be used to prevent adherence of the heated metal tip to the polyethylene surface.

This displacement procedure is being used routinely in our Biochemical Laboratories, as incorporated into our method for determining amino acids.<sup>1</sup> FIGURE 2 shows the system we are using for cholesterol determination and the use, which has been satisfactory, of the displacement container for continuously adding a sulfuric acid-ferrous chloride stream to the flowing reagents. FIGURE 3 shows a close-up of the container in the same system.

Another limitation of the AutoAnalyzer system with which we were confronted was the size of sample required. Many of the biological fluids we wish to analyze are not readily available from our small experimental animals in the



FIGURE 2



FIGURE 3

required 0.3- or 0.4-ml. quantities. This necessitated a dilution prior to aspiration of the sample into the analysis system. After this dilution was made, the sensitivity of the analytical methods and the physical limitations of the standard AutoAnalyzer system did not permit adequate expansion of the standard curve and the samples on the recorder.

A variety of changes was made in attempts to magnify the number of chart units of deflection for a given dilute solution of glucose. After arriving at a method for determining micro quantities of glucose similar to the one reported by Technicon Controls Corporation, we decided to investigate modifications of the electronic circuitry of the colorimeter and recorder for increasing sensitivities. It was found experimentally that adequate expansion could be obtained by placing a Heathkit resistance substitution box in series with the negative side of the reference photocell.

Utilization of this finding takes advantage of the characteristic stability of the colorimeter circuit of the AutoAnalyzer. Since both photocells view the same light source, the 100 per cent transmission position for the slide wire contact, once established, remains fixed. It is effectively independent of the relative potential of the zero end of the slide wire. Thus it is possible to establish the zero and 100 per cent transmission settings for this signal-difference measuring circuit in the conventional manner, and then raise the recorder zero per cent transmission potential relative to the 100 per cent T potential by inserting a resistance in series with the negative side of the reference photocell.

If, as in the case of the micro-glucose method, an absorbing reagent is pumped through the colorimeter so that the per cent transmission of the light incident on the sample cell is reduced to, let us say, 90, then the potential at the recorder "zero" may be raised by inserting a resistance of sufficient size that the potential is equivalent to the output of the sample photocell at the 90 per cent transmission level; then the recorder span represents actually 90 to 100, rather than 0 to 100, per cent transmission.

In practice, it is found necessary also to attenuate the reference photocell output simultaneously by putting a smaller aperture in the light beam. This effectively lowers the potential difference across the slide wire  $R$  plus insert  $R$ . It consequently tends to reduce the amount by which the recorder "zero" is raised to a greater extent than it reduces the recorder span, because the recorder  $R$  is small in comparison with the insert  $R$ . The adjustment with smaller apertures tends, then, to bring the recorder pen back on scale when it has been sent off scale by insertion of a resistance that makes the slide wire cover a smaller transmission range. Since the decrease in aperture size does not greatly affect the recorder span, the net result is an increase in sensitivity of the recorder for solutions having high transmission, that is, low concentrations of the absorbing component.

The precise response characteristics of this modified circuit have not been defined; they are not necessary to the utilization of the instrument as long as sample data are evaluated with respect to a calibration curve established with use of standards evaluated under the same operating conditions.

FIGURE 4 shows the relative effects of changing the resistance and the aperture on a micro-glucose standard curve (1-10 mg./per cent). It may be seen



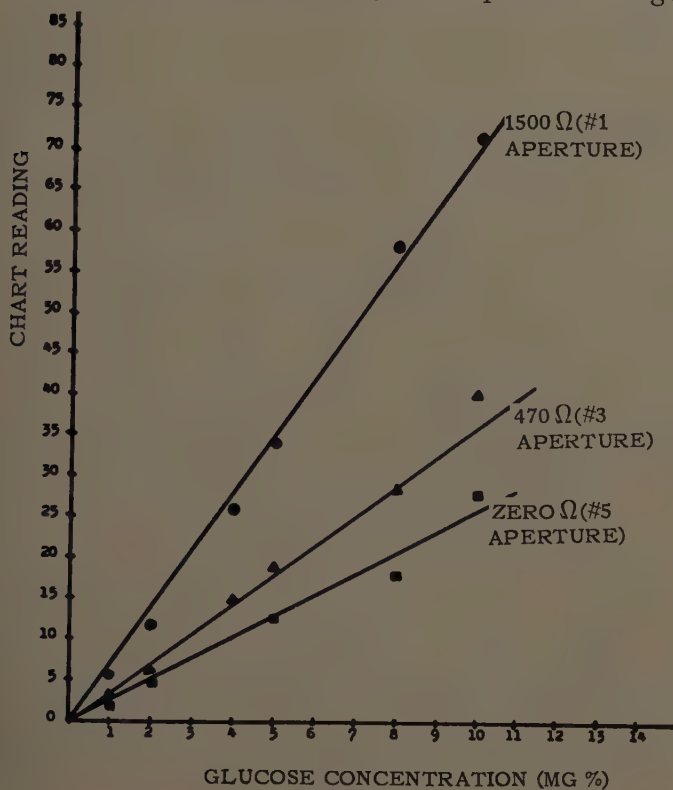


FIGURE 4

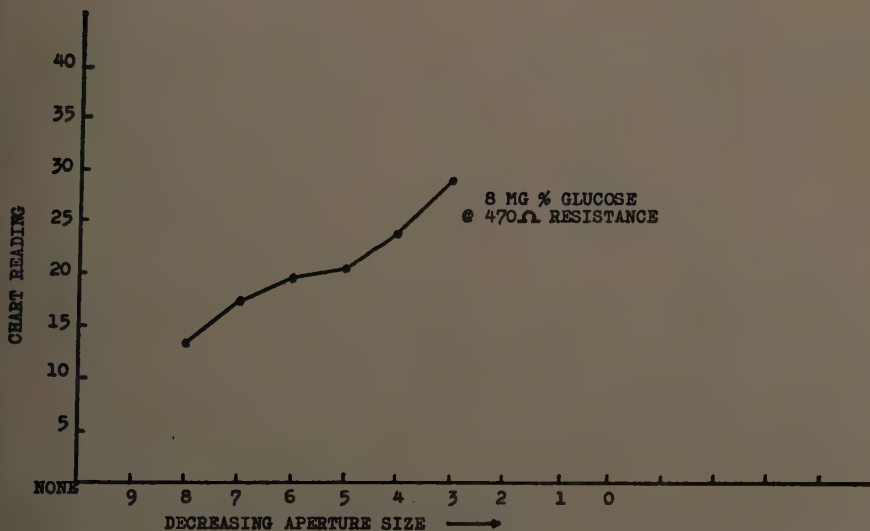


FIGURE 5

from this graph that, as the resistance is increased and the aperture size decreased, the sensitivity of the measuring circuit is increased substantially. The effect of decreasing aperture size upon sensitivities may be seen in FIGURE 5. TABLE 1 illustrates the ranges of aperture sizes available for each resistance.

The circuit diagram (FIGURE 6) illustrates the placement of the resistance substitution box in the AutoAnalyzer circuit. The area enclosed by the dotted

TABLE 1  
RANGES OF APERTURES AVAILABLE FOR EACH RESISTANCE

$R\ (\Omega)$	Aperture size	$R\ (\Omega)$	Aperture size
0	-5	680	6-2
330	9-3	1000	3-2
470	8-3	1500	1

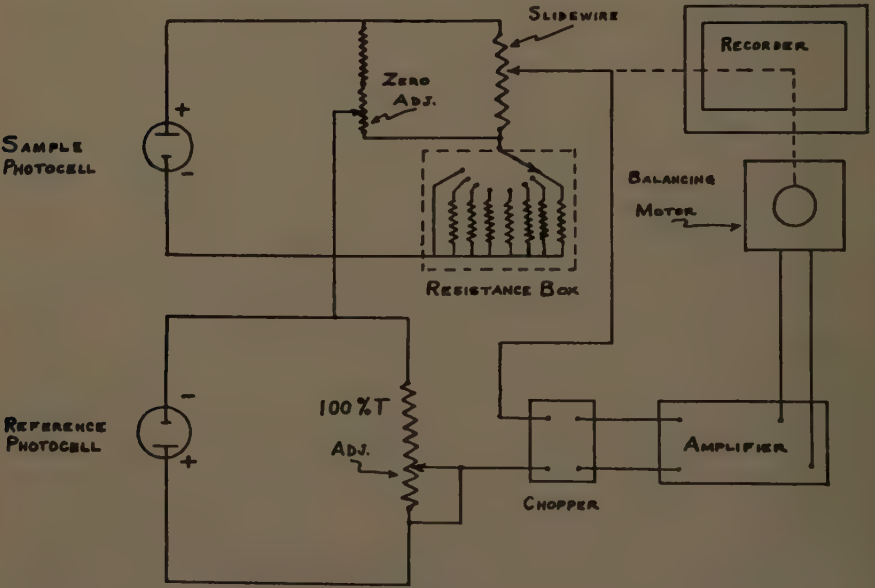


FIGURE 6

line in the circuit diagram represents the position at which the circuit is broken and the resistance is placed in series with the negative side of the reference photocell. The circuit is broken at the wiring plate in the recorder, and the insertion of the resistance substitution box is made.

Resistance substitution boxes have been installed in all of our AutoAnalyzers. They are being utilized routinely to expand the standard curves for all methods. This enables us to use the entire strip chart; therefore, we can read the chart with less error because the intrinsic reproducibility characteristics of the AutoAnalyzer have not been altered substantially. If the intrinsic error does

not increase as the number of chart units increases, the percentage of error in reading the chart is decreased.

In summary, simple instrument modifications, together with alterations for sample- and reagent-handling procedures, have permitted extension of the range of applications of the AutoAnalyzer. The displacement technique<sup>1</sup> has been improved and its applicability extended. Sensitivity of the system for certain types of samples has been considerably increased.

#### *Acknowledgments*

We acknowledge the able assistance of Edward J. Reed, who performed many of the evaluation tests for the modified instrumentation, and of William Kruse, who prepared the illustrations used in this paper.

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# AUTOMATION OF THE MICROBIOLOGICAL ASSAY OF ANTIBIOTICS WITH AN AUTOANALYZER INSTRUMENTAL SYSTEM

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## *Introduction*

The classic methods for microbiological analysis of antibiotics require tedious and time-consuming multiple-step manual procedures. The ever-increasing load placed on the laboratories since the advent of antibiotics has demanded automation. Attempts at automation have been made by mechanization of individual steps. Complete automation on a continuous basis was not feasible until the development of the AutoAnalyzer\* instrumental system.<sup>1,2</sup> The basic principle of the AutoAnalyzer, analysis via a continuously flowing reagent system, suggested a means of assay wherein the inhibitory effect of antibiotics could be impressed upon a continuous stream of metabolizing microbial cells. The instrumental system would have increased efficiency with freedom from human error and would operate with a minimum of supervision. This report describes the AutoAnalyzer system used to demonstrate the feasibility of this objective.

## *Methods and Results*

*Turbidimetric method.* Turbidimetric assay methods are based on the inhibition by antibiotics of the growth of bacteria or yeast. The antibiotic and cells are mixed together in a nutrient medium composed of glucose, partially hydrolyzed protein, growth factors, and salts. After incubation at 37° C. for 3 to 24 hours the cell density is measured with a suitable photometer.

By feeding continuous streams of inoculum and nutrient medium into the AutoAnalyzer and by periodically introducing antibiotic solutions, we were able to obtain dose-response curves for the antibiotics tetracycline, streptomycin, and neomycin. The microorganism used was the bacterium *Klebsiella pneumoniae*.

FIGURE 1 is a photograph of the instrumental system used. For illustration, the double dialyzer and incubation coil are shown raised from their 37° C. baths. FIGURE 2 is a schematic diagram of this system.

Individual 2-ml. samples of antibiotic were placed in the sampler cups. The sampler presented a different sample to the system every 3 min. The proportioning pump withdrew the sample at the rate of 1.2 ml./min.; consequently, after 1.5 min. the sample was exhausted and only air was pumped for the remaining 1.5 min., serving to separate successive samples.

The suspension of bacterial cells (about 0.2 vol. per cent or  $10^7$ /ml.), maintained in suspension by a magnetic agitator, was kept cold to preserve viability and to prevent growth. Since the heat generated by the magnetic stirrer

\* Technicon Instruments Corp., Chauncey, N. Y.



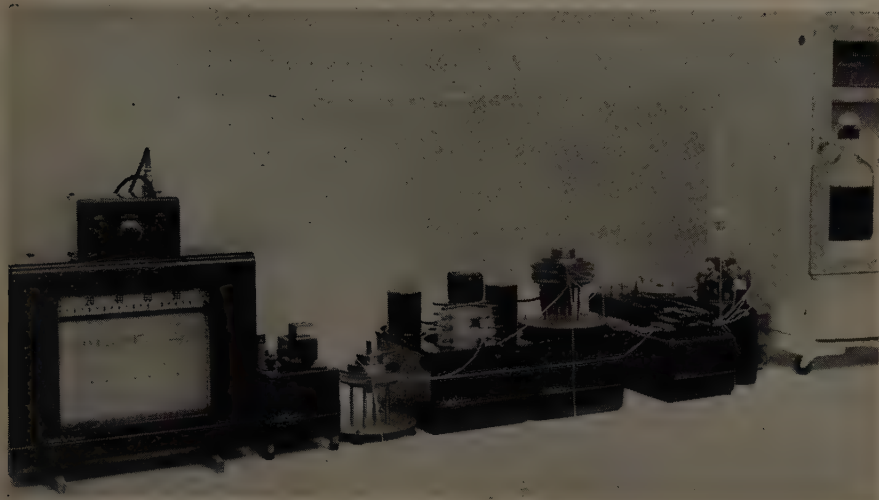


FIGURE 1. Instrument system for turbidimetric method.

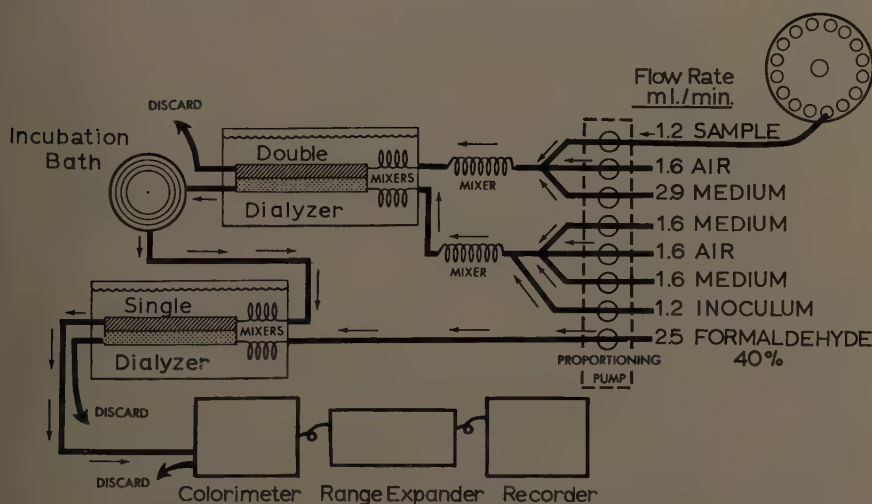


FIGURE 2. Schematic of automatic microbiological determination of antibiotics via measurement of growth density.

exceeded the capacity of the refrigeration unit, it was necessary to use an ice bath to maintain a constant temperature of  $6^{\circ}\text{C}$ .

Nutrient medium A\* was maintained under refrigeration to prevent growth of contaminants. Tween 20\* was added to decrease the surface tension.

The proportioning pump mixed and conveyed two streams: one composed of sample with nutrient medium as diluent, segmented with air; the second composed of bacterial suspension and nutrient medium, likewise segmented

\* See TABLE 1.

with air. For segmentation of the latter stream a modified bubbler was used to make large bubbles that did not break up during their flow through the deep-groove dialyzers and incubation coil. The modified bubbler differed from the conventional bubbler in that the diameter of its lumen was enlarged to 4 mm.

The two streams were passed through opposing sides of the double, deep-groove dialyzer to permit the antibiotic to dialyze into the stream containing the bacteria. The stream containing the spent sample was discarded and the other stream, containing bacteria and sample, was pumped through an incubating coil consisting of 75 feet of 0.125-inch diameter polyethylene tubing. As the stream emerged from the incubating coil, it was dialyzed against formalin to kill the bacterial cells.

Forty minutes after entering the system, the portion of the stream that had been influenced by the antibiotic entered the 4-mm. flow cuvette, which

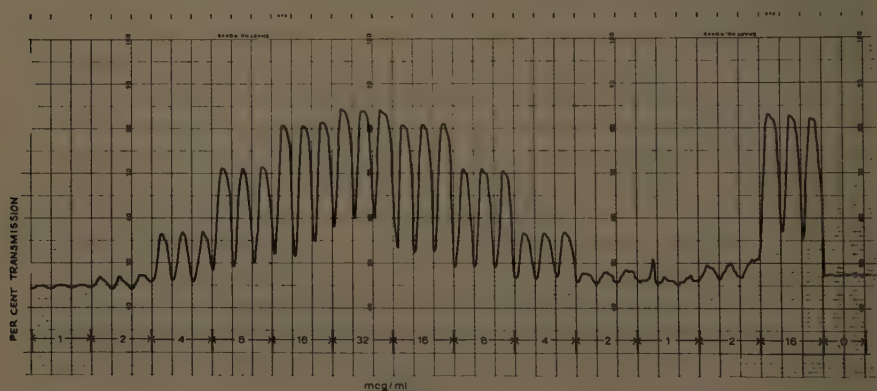


FIGURE 3. Tetracycline dose-response curve.

was modified so that the liquid level in the viewing chamber was raised 4 mm. Also, the connecting member between the precollection chamber and the viewing chamber was constricted to provide hydraulic damping. The resulting turbidity was recorded as absorption of light at 543  $m\mu$ . During this short incubating period, the bacterial numbers increased 70 per cent, an amount insufficient to give a full-scale deflection of the recording system. To increase the readability of the chart, the amplitude of the deflection was increased four-fold with the range expander.

After each 2- to 3-hour run, the entire system was flushed for about 20 min. to remove cells of *K. pneumoniae* and any contaminating microbes that might have accumulated on the inner surface of the tubing. A disinfecting and cleaning mixture, 25 per cent concentrated hydrochloric acid in nutrient medium, was used for flushing. Since prolonged exposure to the acid medium reduced the life of the manifold tubing, the contents of the system were replaced with an antiseptic mixture, 20 per cent ethanol in nutrient broth, when it was necessary to shut down the system.

*Turbidimetric results.* Samples containing 1 to 32  $\mu$ g. tetracycline/ml. in

water were introduced into the AutoAnalyzer system. The turbidimetric response is shown in FIGURE 3. Replicate samples introduced into the system showed a standard deviation of  $\pm 0.6$  per cent transmission, or about  $\pm 5$  per cent potency. The dose-response curve of these samples is illustrated in

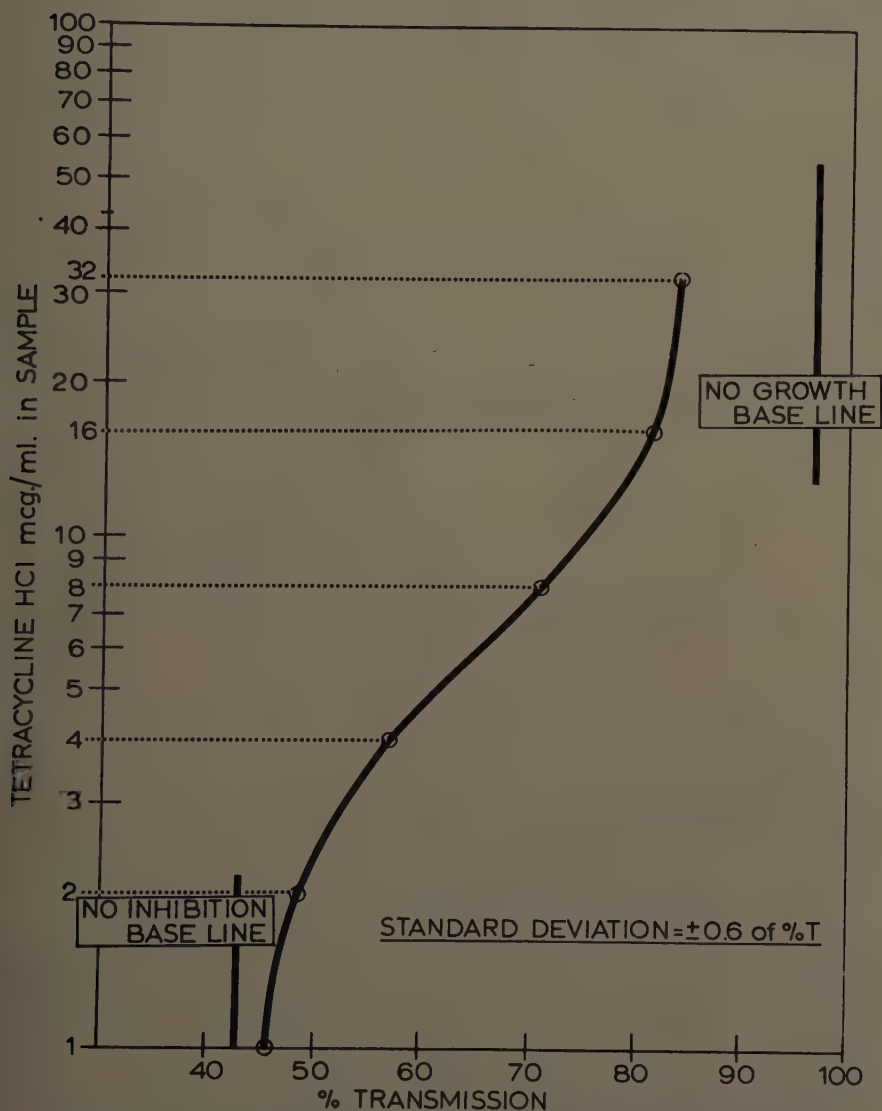


FIGURE 4. Tetracycline-HCl dose-response curve via measurement of cell density.

FIGURE 4. Full growth (no inhibition of the test organisms) gave a transmission reading of 43 per cent when the colorimeter was adjusted to give a reading of 97 per cent for complete inhibition (no growth).

Neomycin and streptomycin showed precision and dose-response curves

similar to those for tetracycline, but higher concentrations were needed. For neomycin, the sensitive region was 150 to 1200  $\mu\text{g./ml.}$  and for streptomycin, 250 to 4000  $\mu\text{g./ml.}$

*Respirometric method.* Metabolizing bacterial and yeast cells produce carbon dioxide, while antibiotics inhibit this action. By feeding into the AutoAnalyzer continuous streams of inoculum with nutrient medium and sequentially introducing therein different levels of antibiotic, and by continuously measuring the carbon dioxide produced, we obtained dose-response curves for the antibacterial antibiotics tetracycline, streptomycin, and neomycin and the antifungal antibiotics nystatin and amphotericin B. FIGURE 5 is a schematic diagram of the system used.

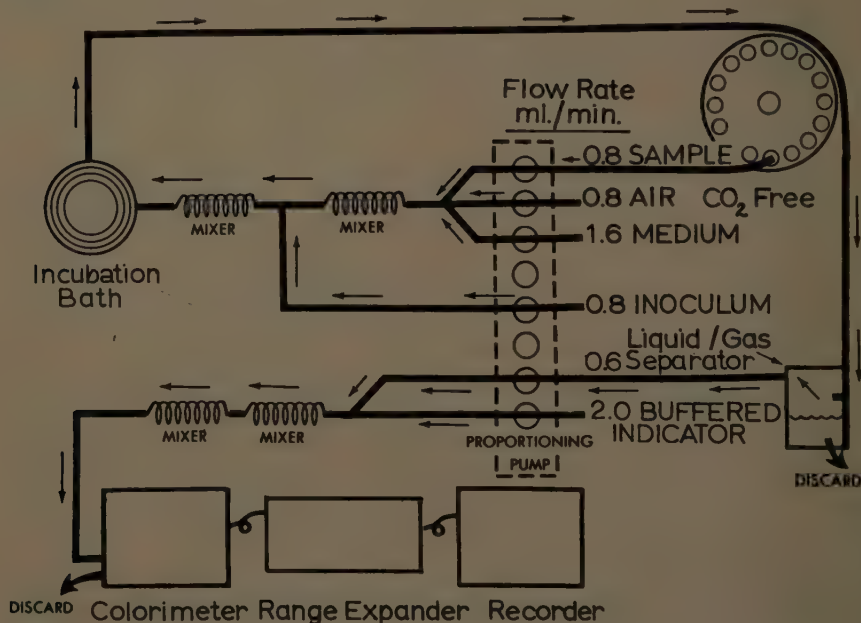


FIGURE 5. Automatic microbiological determination of antibiotics via measurement of respiratory  $\text{CO}_2$ .

For tetracycline, neomycin, and streptomycin a 1 per cent suspension of *Escherichia coli* in 0.1 *M* phosphate buffer (*pH* 6.6) was used with nutrient medium B.\* For nystatin and amphotericin B, a 1 per cent suspension of the yeast *Saccharomyces mellis* in the same buffer was used with nutrient medium C.\*  $\text{CO}_2$ -free air was used for segmentation of the stream and for venting reagent bottles. The samples and sample lines were exposed to atmospheric air.

Nutrient medium and sample streams were combined, segmented with air, and mixed. The bacterial or yeast cell suspension was connected to the sample-nutrient stream and the combined mixture was passed through a second mixing coil. Twenty minutes after entering the glass coil in the 37° C. water bath, the stream was pumped to a liquid-gas separator, where the  $\text{CO}_2$ -en-

\* See TABLE 1.



riched air was separated from the liquid phase. An aliquot of the air phase was removed by the proportioning pump used for segmenting the alkaline buffer-indicator stream (TABLE 1) and the remaining air was then used to expel the liquid from the separator. The segmented stream of alkaline buffer with indicator was passed through two mixing coils to allow equilibration of the CO<sub>2</sub> in the air phase with the alkaline buffer-indicator. The color intensity of the indicator was inversely proportional to the amount of CO<sub>2</sub> present. The color intensity at 555 m $\mu$  was measured as the stream passed through the 4-mm. flow cuvette of the colorimeter. To obtain a full-scale deflection of the recording system, the range expander was used to increase the amplitude of the deflection twofold.

Before the sample was admitted to the system, a base line had been established for uninhibited CO<sub>2</sub> production by use of a constant flow of distilled water in the sample line. As antibiotic solutions were introduced into the

TABLE 1

Tween 20* (0.25%) was added to all nutrient media and cell suspensions.	
Nutrient medium A:	Penassay broth†
Nutrient medium B:	1.75% Penassay broth, dehydrated
	0.5% yeast extract
	1.0% Tryptone†
	0.9% Casitone†
Nutrient medium C:	2.0% glucose
	0.5% yeast extract
	1.0% sodium citrate
	0.1% monopotassium phosphate
	0.1% dipotassium phosphate
Alkaline buffered indicator:	3 ml. 1% phenolphthalein in methanol
	0.5 ml. 1.0 M sodium carbonate
	1.0 ml. 1.0 M sodium bicarbonate
	Make to 1 l. with CO <sub>2</sub> -free distilled water

\* Atlas Powder Co., Wilmington, Del.

† Difco Laboratories, Detroit, Mich.

stream in sequence, they inhibited the growth of the organism, as measured by carbon dioxide production.

At the end of each day's run all lines were flushed with 2 *N* hydrochloric acid, followed by distilled water.

*Respirometric results.* Tetracycline samples were introduced into the system at concentrations of 2.5 to 54  $\mu$ g./ml. in water. The responses are shown in FIGURE 6. Analysis of replicate determinations with these same concentrations showed a standard deviation of  $\pm 0.5$  per cent transmission or  $\pm 3$  per cent potency. A plot of this dose response is illustrated in FIGURE 7.

Other antibiotics gave dose-response curves similar to those for tetracycline. TABLE 2 shows the sensitive region for each antibiotic under the conditions of this method.

### Discussion

Although we have successfully measured antibiotic activities by the use of two separate modalities, cellular growth and cellular respiration, some prob-

lems remain to be solved before an effective tool for analysis is achieved. The major problems were dialysis efficiency, internal pressure, maintenance of cells in uniform suspension, sample discrimination, and drift. Experience gained from initial experiments on the development of a turbidimetric method was of value in our later experiments on the development of the respirometric method.

*Dialysis.* Most antibiotics, due to their large molecular weight, dialyze

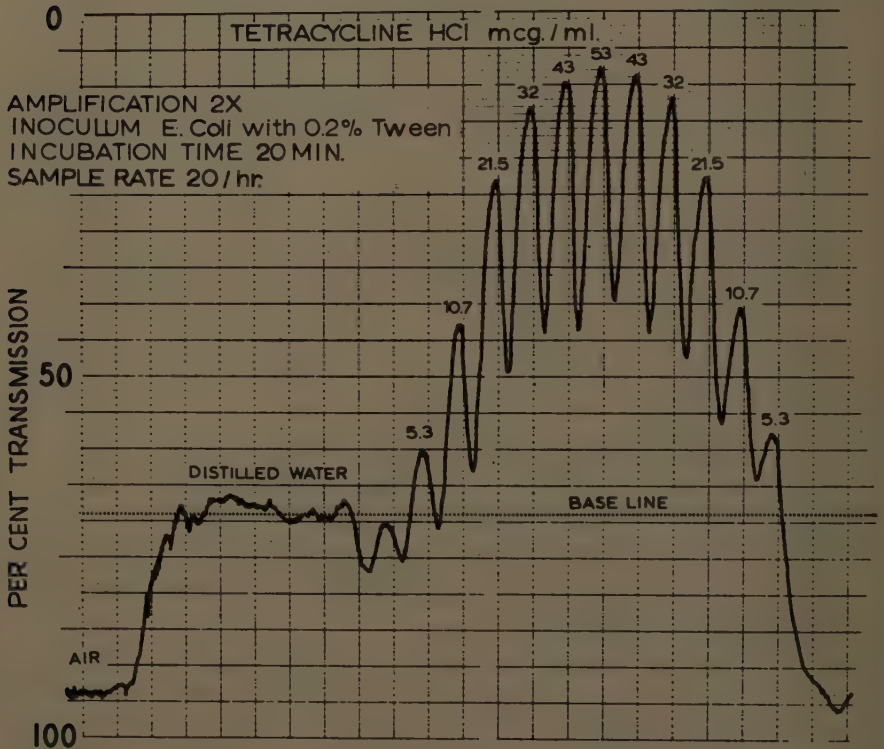


FIGURE 6. Response of *Escherichia coli* to tetracycline via measurement of respiratory  $\text{CO}_2$ .

poorly through a cellophane membrane. For example, with the turbidimetric method, 1 per cent of the neomycin in the sample stream passed into the recipient stream. Similar results were obtained with streptomycin and tetracycline. The small amount dialyzed limited the use of this system to relatively concentrated antibiotic solutions. The sensitivity of the method could be materially increased by dialyzing under pressure or by by-passing dialysis.

Under the conditions described for the turbidimetric method, dialysis was necessary because by-passing the double dialyzer resulted in an erratic response. With the respirometric method dialysis was not necessary.

*Internal pressure.* In early turbidimetric work, a 220-foot polyethylene coil

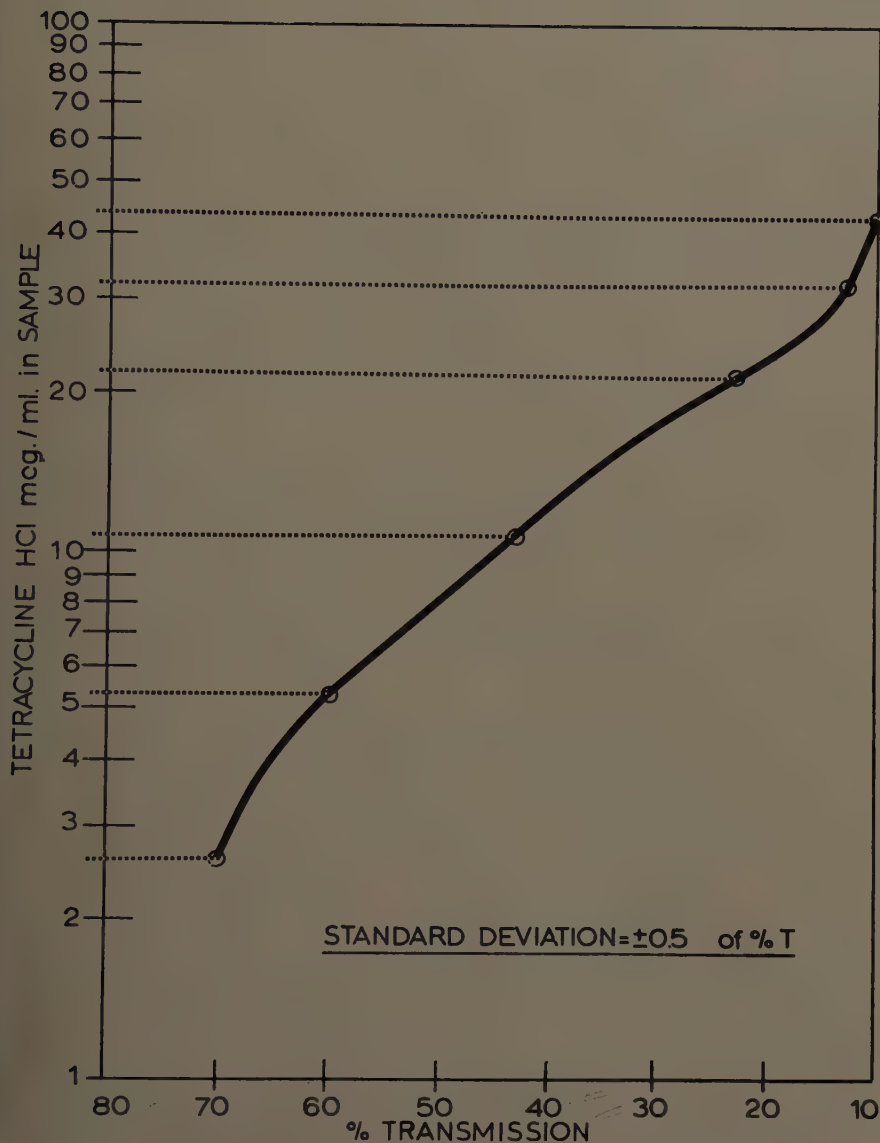


FIGURE 7. Curve of tetracycline-HCl dose response via measurement of respiratory  $\text{CO}_2$ .

TABLE 2  
SENSITIVITY OF RESPIROMETRIC SYSTEM TO ANTIBIOTICS

	Sensitive range, * $\mu\text{g.}/\text{ml.}$	
	Low	High
Streptomycin $\text{SO}_4$	300	4000
Neomycin $\text{SO}_4$	100	3000
Tetracycline HCl	2	50
Nystatin	2	10
Amphotericin B	0.2	2

\* Concentration in sample cup.

0.066 inches in diameter was used to give a 35-min. incubation when the fluid was pumped at 5.6 ml./min. The internal pressure, 12 to 15 cm. Hg, frequently ruptured the cellophane membrane or broke one of the joints in the manifold. Pressure changes due to use of different fluids (nutrient media and flushing solutions) caused severe irregularities in flow rate. This condition was alleviated without alteration of the incubating time, by use of a shorter, wider coil (75 feet long, 0.125 inch in diameter). The pressure developed in this coil was only 2 cm. Hg. For the respirometric system, a 40-foot glass coil, 0.090 inch in diameter, was used to give a 20-min. incubation at a pumping rate of 3.8 ml./min. Pressure was no problem with this system.

*Maintenance of microbial cells in uniform suspension.* The microbial cells tended to settle and adhere to the surface of the tubing. This effect was negligible in the respirometric system. In general, the problem was greater with the large yeast cells than with the smaller bacterial cells. Settling in the inoculum reservoir was prevented by magnetic agitation. A small tube, 0.034 inch in diameter, was used for the inoculum delivery line to maintain a high velocity. The addition of Tween 20 to both the inoculum suspension and nutrient medium greatly decreased the tendency of cells to adhere to the surface of the tubing in the turbidimetric method, and eliminated it in the respirometric method.

*Drift.* Both methods exhibited a constant increase in either turbidity or carbon dioxide produced (see FIGURE 3). This drift was smaller in the respirometric system. The rate of drift varied with conditions from 1 to 10 per cent per hour and was sufficiently uniform to justify a linear correction of the resultant responses. Further work is necessary to eliminate this problem, however.

*Sample discrimination.* Barely adequate separation between samples was obtained with both methods at a sampling rate of 20 per hour. The influence of one sample upon the following sample was particularly noticeable when the concentrations of each were widely different, for example, greater than eight-fold for the turbidimetric system and greater than twofold for the respirometric system.

The promising results obtained with the turbidimetric and respirometric methods of measuring the inhibitory effect of antibiotics on bacteria and yeast justify further development and the exploration of other methods which may be adapted equally well to the AutoAnalyzer. Other indicators of metabolism might be the rate of utilization of glucose or amino nitrogen or the rate of production of urea or ammonia.

### Conclusions

Automation of microbiological assay procedures is feasible. This was demonstrated with the AutoAnalyzer by carrying out continuous analyses for antibacterial and antifungal antibiotics by turbidimetric and respirometric methods.

The conditions that must be rigorously controlled to yield an accurate method include dialysis efficiency, internal pressure, maintenance of cells in suspension, sample discrimination, and drift.



A better understanding of these and related problems will lead to the development of efficient automated instrumental systems, not only for microbiological assays for antibiotics and growth factors, but also for screening or evaluation of analogues or derivatives of microbiologically active compounds. Also, the study of microbial physiology should be possible with this instrument.

#### *Acknowledgment*

We acknowledge the valuable assistance of Joseph Gentile and Herbert Stander.

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# NITROGEN DETERMINATION BY A CONTINUOUS DIGESTION AND ANALYSIS SYSTEM

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The analysis of nitrogen has been of great concern to various workers in the field of biology and chemistry, and a great deal of effort has been spent in the development of methods for it. Moreover, the analysis of nitrogen yields an indirect measure of the composition of such compounds as proteins.

The classic analysis for nitrogen involves oxidation or reduction by digestive procedures. Such procedures are based on the original work of Johan Kjeldahl between 1883 and 1888. Kjeldahl employed in his initial investigations a digestive mixture of sulfuric and phosphoric acids to decompose many organic substances.<sup>1</sup> Essentially, the Kjeldahl procedure involves oxidation of organic matter, giving rise to carbon dioxide and water when boiled with concentrated sulfuric acid, the latter then being reduced to sulfur dioxide. The nitrogen present is converted to ammonium sulfate unless it was initially present as azo or nitro derivatives. In this case it is first treated with a mixture of salicylic acid and sulfuric acid, and the nitrated derivative thus formed is reduced with thiosulfate to an amino derivative. After complete destruction of the organic matter, an excess of sodium hydroxide is added to the sulfuric acid, and the liberated ammonia is distilled into a measured volume of a standard acid. Titration of the excess or residual acid with a standard alkali, using a suitable indicator, permits quantitation of the nitrogen content.

Nitrogen also can be analyzed in organic compounds by conversion to ammonia followed by hypobromite oxidation of the ammonia to nitrogen. The nitrogen evolved is measured in gaseous form<sup>2</sup> or quantitated via a mass spectrometer.

Many digestion mixtures have been proposed for use with the classic Kjeldahl method, and a host of additive aids to oxidation, metal catalysts, and substances to raise the boiling point have been suggested and employed. Some of these mixtures have very specific applications, and others are simply the result of a specific scientist's opinion that his mixture afforded superior digestive efficiency. The essence of the mechanism remains the same, however, regardless of the digestive mixture. Essentially, the Kjeldahl procedure consists of the introduction of a sample into a suitable flask with an appropriate digestive solution, the prolonged boiling of the organic matter to oxidize the carbon and hydrogen with reduction of the sulfuric acid to sulfur dioxide, and the subsequent reduction of the nitrogenous matter to ammonia.<sup>3</sup> This procedure is both tedious and cumbersome; moreover, additional manipulative operations must be carried out in order to arrive at a quantitative answer.

The stumbling block in automating the nitrogen analysis of organic compounds on the basis of the principles established by Kjeldahl was the digestive phase. It was necessary to provide a means for continuous introduction and removal of the digestive mixture and intermittent or continuous introduction of the material to be digested. A suitable time lag had to be provided so that

phases such as reduction and oxidation could occur. In turn, it was necessary to isolate each of these phases in the digestive procedure from the previous phases and also from newly introduced sample material that was beginning its digestive process. Thus the use of a boiling flask with a constant overflow system in which the digestive mixture could be maintained at elevated temperatures during boiling would not serve the purpose. It became evident, then, that multiple and repetitive isolated digestions must be carried out while the digestion fluid was carried forward and collected at some point beyond the digestion phase where it could be assessed continually by some suitable means.

The availability of an automatic analyzing system<sup>4,5</sup> provided the means to quantitate the final product of the classic Kjeldahl procedure, that is, the liberated ammonia present in a digested sample. The multiple proportioning pumps available in this system were used to provide the means to supply continuously the digestion mixtures to the digester, to present the sample continuously or intermittently and, finally, to remove an aliquot of the digested end products for final mensuration.



FIGURE 1. Digestion vessel. *A*, precollection groove. *A*<sub>1</sub>, digested sample collection groove. *A*<sub>2</sub>, excess waste fluid collection groove. *A*<sub>3</sub>, digestion grooves.

The digestion module was still a problem. A digestion vessel consisting of a glass tube with helical indentations was conceived. By rotating this tube, liquid introduced at one extremity would be conveyed as individual puddles or segments to the other extremity to be removed for further processing. FIGURE 1 illustrates precisely the details of such a digestion vessel. The length of this digestion vessel is about 20 inches, the over-all length being approximately 23 inches. A precollection groove *A* is situated near the entrance of the vessel and 2 similar grooves *A*<sub>1</sub> and *A*<sub>2</sub> are provided at the outlet of the system. The diameter of the vessel is approximately 1.7 inches with an i.d. depth of the grooves *A*<sub>3</sub> of approximately  $\frac{1}{16}$  inch. The i.d. width of the grooves is approximately  $\frac{1}{4}$  inch. The extremities of the tube are slightly tapered to prevent condensed moisture from dripping off the end of the tube. The introduction and aspiration pipettes provided fit within the cavity or depression of the single circular groove *A* at the entrance end and the dual circular grooves *A*<sub>1</sub> and *A*<sub>2</sub> at the outlet. The vessel is constructed entirely of Pyrex glass.

FIGURE 2 shows the digestion vessel *A* placed within a split-combustion tubular-type furnace. The vessel is mounted at either extremity on two metal disks (wheels) *B*, which support it within the lumen *C* of the tubular furnace. These wheels rest in turn upon two additional supporting wheels *B*<sub>1</sub> that are

motor-driven by a variable-speed voltage-controlled motor permitting adjustment of the speed of rotation and thus of the duration of the digestive phase. The faster the tube is rotated, the shorter the time allotted to the digestion of the specimen. An electronic-type temperature control and indicator is provided that is adjustable from room temperature to approximately 700° C. Suitable temperature and speed controls with appropriate "on" and "off" switches are provided. FIGURE 3 illustrates the over-all appearance of the unit. It will be noted that the rotating wheels at each extremity have been provided with covers for protection against accidental splashing with digestion

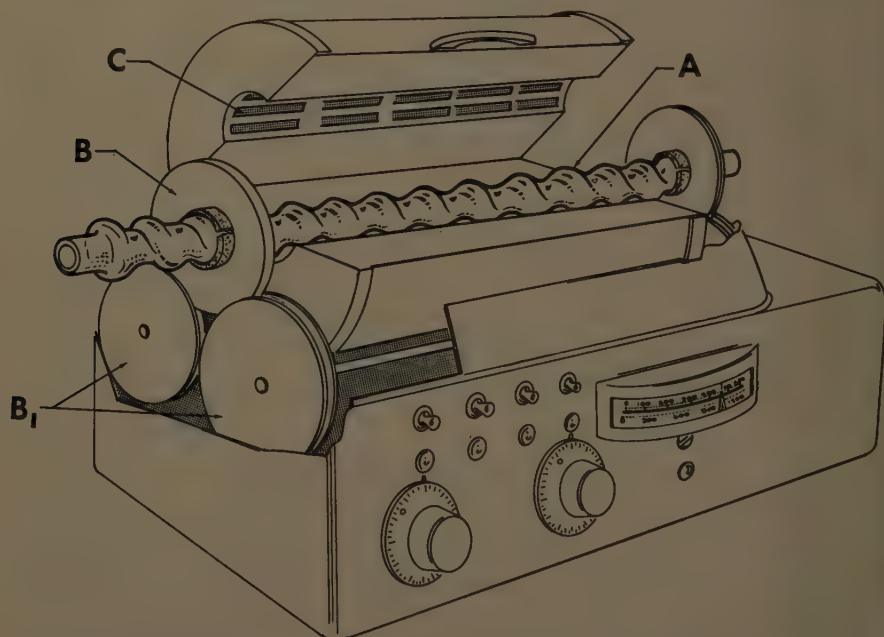


FIGURE 2. Continuous-digestion module. *A*, digestion tube. *B*, digestion tube support wheels. *B*<sub>1</sub>, motor-driven wheels. *C*, furnace lumen.

mixtures. The reagents employed in this system are listed in detail in the *Appendix*.

The flow diagram for the procedure employing this automated system may be seen in FIGURE 4. Two multiple proportioning pumps are used. Pump No. 1 delivers through appropriate tubes the desired volume of digestion solution, as well as continuously or intermittently the sample aspirated from a sampling module, vessel, or continuous process. The combined streams of sample and digestion solution are fed into the right-hand extremity of the digestion vessel. Continuous rotation carries the combined streams through the digestion phase. As indicated previously, the degree of digestion may be increased or decreased by varying temperature and time of passage through the furnace. On emergence from the left-hand side of the digestion vessel,



the aspirating pipette situated in the first or inner groove ( $A_1$ , FIGURE 1; see enlarged detail in FIGURE 4) continuously aspirates an aliquot of the digestion solution which may or may not contain a digested sample. The excess digestion fluid carried forward is continuously removed by a water aspiration pump

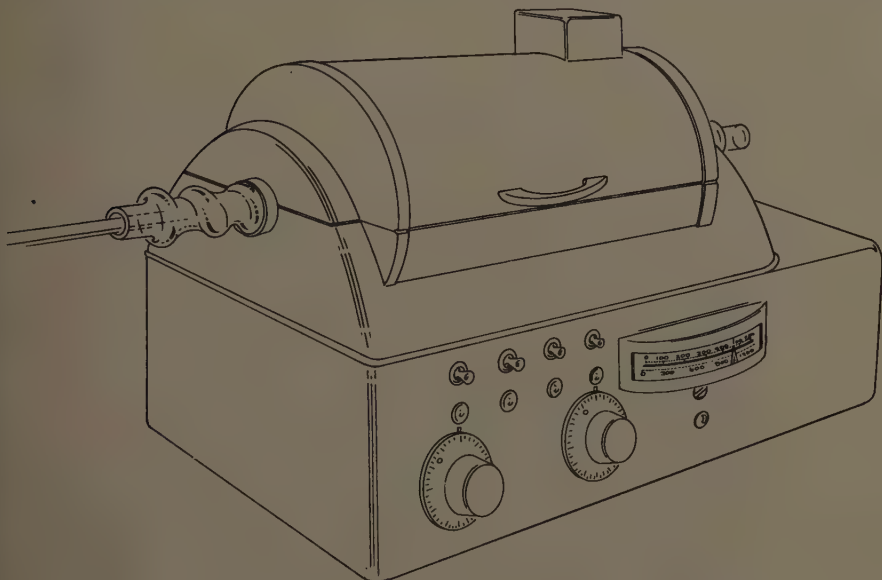


FIGURE 3. Digestion module.

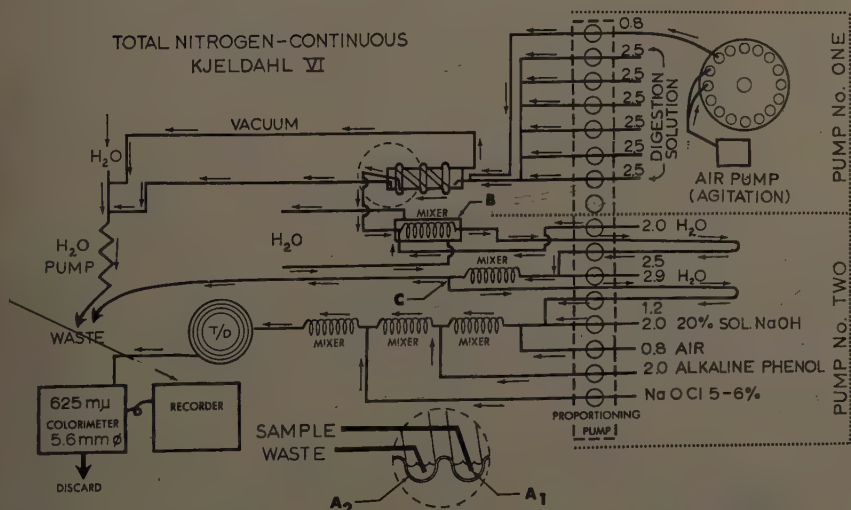


FIGURE 4. Flow diagram for automatic nitrogen determination via continuous digestion.  $A_1$ , sample aspiration groove.  $A_2$ , excess digestion fluid aspiration groove.  $B$ , jacketed mixing coil.

and sent to waste from the last or outer circular groove in the digestion vessel ( $A_2$ , FIGURE 1). The large volumes of fumes and water vapor evolved in this digestive process are aspirated continuously by a suction tube placed at the right-hand end of the digestion vessel; this, too, is accomplished via a water pump and the resulting condensed gases are sent through to waste. Due to the corrosive nature of the fumes, the water aspiration pump is constructed entirely of glass.

At the elevated temperatures at which the unit is normally operated (400 to 600° C.), it must be realized that the digestion solution emerging from the outlet of the vessel is highly concentrated; indeed, it is anhydrous. The as-

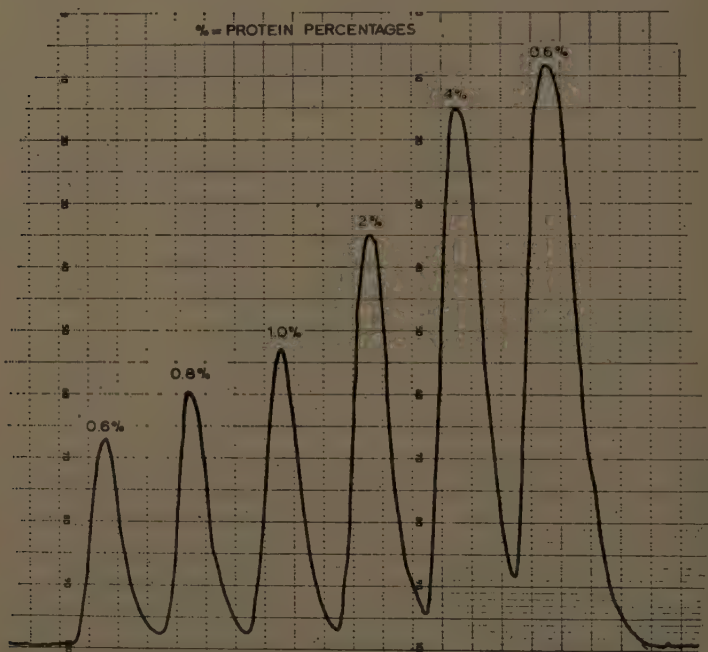


FIGURE 5. Typical recording of ammonium sulfate standards.

pirated aliquot of digestion solution is diluted and mixed in a jacketed mixing coil ( $B$ , FIGURE 4), suitably cooled with running tap water. The diluted and cooled digestion solution is aspirated by pump No. 2 and further diluted with additional water and mixed; then an aliquot of this diluted digestion solution is removed through a T connection ( $C$ , FIGURE 4), aspirated, and introduced into a stream of 20 per cent sodium hydroxide solution. Alkaline treatment of the digestion solution results in neutralization and liberation of ammonia from the ammonium sulfate derived from the sample. Ammonia-free air is introduced for segmentation; the solution then is sent through a mixing coil, and a solution of alkaline phenol followed by mixing; then the introduction of 5 to 6 per cent sodium hypochlorite solution gives rise to an intense blue product believed to be indophenol or a closely related substance.<sup>6-8</sup> The resultant

indophenol-blue reaction takes place immediately after the solution is passed through a heating coil at 95 to 100° C. or after suitable time delay, as in this particular case. The resultant reaction is measured at 625  $m\mu$ . A 5.6-mm.  $\phi$ -flow cuvette normally is employed.

It must be understood clearly that the end product of the digesting phase, ammonium sulfate, need not be measured by this particular analytical procedure. A titrimetric approach could be employed, with a buffered indicator, to

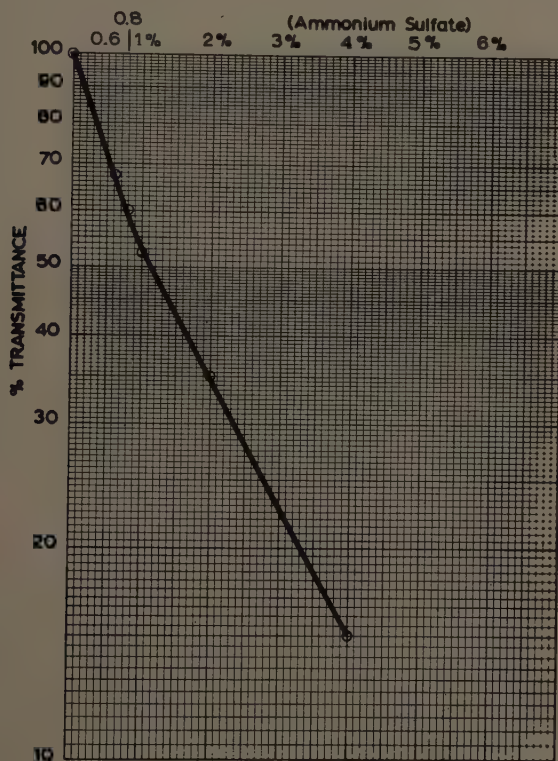


FIGURE 6. Semilog plot of ammonium sulfate standards.

quantitate the resultant ammonia, furthermore, many other ammonia reagents are equally suitable. The ninhydrin reagent has been employed successfully.

FIGURE 5 is a recording of a typical response to ammonium sulfate standards representing percentage protein concentrations.

FIGURE 6 shows the nearly linear response of the previous values plotted on semilogarithmic paper. TABLE 1 shows the result of analysis of yeast cell suspensions in which the classic Kjeldahl determination was performed with copper sulfate and sulfuric acid as the digesting mixture. Methyl purple was used as the indicator and sodium hydroxide for the evolution of ammonia, followed by distillation and titration of the distillate with 0.1 *N* NaOH. The results were converted to read as per cent protein. The values in the first

column were those obtained by the manual procedure. The values in the second column are those obtained by the automated procedure described in this paper. Four of the automated values gave lower results than those achieved by the manual procedure, and 6 gave slightly higher values. FIGURE 7 shows

TABLE 1  
DETERMINATIONS OF PROTEIN NITROGEN IN YEAST SUSPENSIONS\*

Per cent protein (manual method)	Per cent protein (automated method)
46.5	43.3
44.2	47.4
49.5	53.9
48.1	49.9
51.1	49.9
51.1	49.9
38.7	41.9
48.7	49.6
39.7	44.9
52.8	44.9

\* Analyzed yeast suspensions supplied by the Research Laboratories of the Anheuser-Busch Company, St. Louis, Mo.

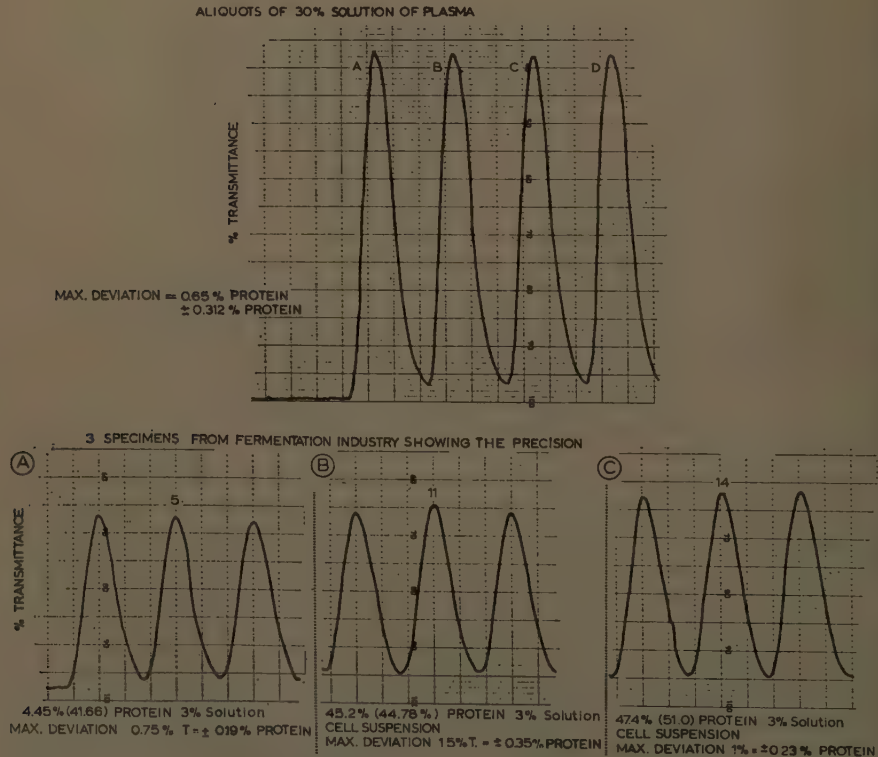


FIGURE 7. Replicas of various samples to show reproducibility.



a series of replicas on both plasma solutions and yeast cell suspensions which, we feel, validates the answers obtained with this automated system. It will be noticed that, in the 3 yeast replicas *A*, the value indicated by the manual procedure was 44.5 per cent. The values obtained by the automated procedure gave 41.66 per cent. The maximum deviation in per cent transmission is 0.75, which is equal to  $\pm 0.19$  per cent protein for the replicas. In *B*, the manual procedure gave 45.2 per cent and the automated procedure, a value of 44.7 per cent. The maximum deviation in per cent transmission is 1.5 or  $\pm 0.35$  per cent protein for these replicas. In *C*, the manual procedure gave 47.4 per cent, whereas the automated method yielded 51.0. Maximum deviation in per cent transmission was equivalent to  $\pm 0.23$  per cent protein. The replicas of the 30 per cent plasma solutions showed a total deviation of 0.62, or  $\pm 0.31$  per cent protein.

In TABLE 1 it will be noted that three of the manual determinations gave 48.1, 51.1, 51.1 per cent, respectively. In each case, the automated procedure gave 49.9 per cent protein, which differed by +3.7, -2.33, and -2.33 per cent, respectively, from these three samples.

### *Conclusion*

It is felt that the development of a continuous-digestion module permits automatic analysis of nitrogenous compounds with reliability and reproducibility unobtainable by conventional methods. At present, the rate of analysis is approximately 10 per hour. It is felt that with improvements in design of the digestion vessel the rate of analysis can be increased considerably. At present, the total time that elapses from the moment the sample is aspirated until the results are recorded may range from as little as 9 min., up to 18 min. The variation in time is due to differences in sample concentration and severity of the digestion procedure required.

Both reductive and oxidative types of digestion can be automated with this instrumental system. It is felt that the mechanization of the digestion phase and the subsequent mensuration of the liberated ammonia will bring about greater precision in analysis of nitrogen from a diversity of sources.

### *Acknowledgments*

I am indebted to George Reinhardt and William Hardwick, of the Research Laboratories of the Anheuser-Busch Company, St. Louis, Mo., for supplying yeast samples analyzed by the classic Kjeldahl procedure, and to R. J. Smith of the Research Department of the Corn Products Company, Argo, Ill., for protein samples.

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*Appendix*

The reagents for continuous determination of nitrogen are made up as follows:

(1) Digestion solution: to 1 l. of 50 per cent v/v sulfuric acid add first 3.0 ml. perchloric acid, stir, then add 4.8 ml. selenium oxychloride; stir and bottle in glass bottles with polyethylene-lined caps.

(2) Twenty-six per cent sodium hydroxide solution.

(3) Alkaline phenol: weigh out 250 gm. phenol (crystalline\*) and place in 2 l. Erlenmeyer flask. Place flask in large vessel with ice cubes or cold running water. While stirring slowly, add 500 ml. 20 per cent NaOH. When the phenol crystals are dissolved, transfer contents of the Erlenmeyer to a volumetric flask and dilute to volume with 20 per cent NaOH. Bottle in amber glass or polyethylene bottles. Proper cooling and stirring during slow addition of the NaOH will give a light-colored solution and a corresponding low reagent blank.

(4) Alkaline hypochlorite solution: weigh out 35.7 gm. calcium hypochlorite (Mathieson HTH, 70 per cent). Dissolve in 300 ml. hot water. Weigh out 40 gm. anhydrous potassium carbonate (C.P.), and dissolve in 200 ml. water. Add 135 ml. anhydrous potassium carbonate solution to the calcium hypochlorite solution, and mix. Heat to exactly 90° C., cool rapidly, and dilute to 500 ml. with water. Filter a small portion and test for calcium ion as follows: to 1 ml. of solution add 2 to 3 ml. potassium carbonate solution and heat in boiling water for a few minutes. The solution should remain clear in the absence of calcium ion. If calcium is present, add more potassium carbonate to the solution and repeat test for calcium. When test is negative, filter and bottle solution in amber containers.

(5) Ammonium sulfate standards to represent per cent protein solutions: prepare a stock solution containing 0.1 gm.  $(\text{NH}_4)_2\text{SO}_4/\text{cc. (ml.)}$ . To make solutions equivalent to the per cent protein concentrations, in the first column below, dilute the amounts of stock (ml.) in the second column up to 100 ml. with water:

0.1	0.754
0.2	1.51
0.4	3.02
0.6	4.52
0.8	6.03
1.0	7.54
2.0	15.10
4.0	30.20
6.0	45.20
8.0	60.30
10.0	75.40

The following formula was used to compute the above:

$$\frac{\text{gm. protein}}{100 \text{ ml.}} \times \frac{1}{6.25} \times \frac{132.146}{28.016} = \text{gm. } (\text{NH}_4)_2\text{SO}_4/100 \text{ ml.}$$

$$\text{The constant factor} = \frac{132.146}{6.25 \times 28.016} = 0.754$$

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\* J. T. Baker, Phillipsburg, N. J.

# A METHOD FOR THE DETERMINATION OF AMMONIA IN BIOLOGICAL MATERIALS ON THE AUTOANALYZER

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Biochemical and biological research often involves experiments requiring ammonia determinations. It is often impossible to determine ammonia directly, owing to the presence of interfering substances. Thus the separation of ammonia by steam distillation, aeration, or diffusion are the tedious, time-consuming techniques usually employed. The use of the AutoAnalyzer\* for ammonia determinations, utilizing dialysis to separate the ammonia from the system under analysis and subsequent colorimetry to determine ammonia, provides an accurate and simple method for handling large numbers of samples.

The method most suitable for the colorimetric determination of ammonia was found to be the alkaline phenol-hypochlorite reaction as described in 1944 by Russell,<sup>1</sup> who found that the sensitivity of the reaction was increased greatly if equivalent amounts of phenol and base were used.

An adaptation of the alkaline phenol-hypochlorite reaction to the AutoAnalyzer is the subject of this report.

## EXPERIMENTAL

### *Preparation of Reagents*

The reagents as presented are essentially those of Russell, except that the concentration of the alkaline phenol has been changed from 2.7 *N* to 0.9 *N*.

#### *Alkaline phenol:*

- (1) Dissolve 83.3 gm. phenol by the addition of 15 ml. distilled water.
- (2) To this solution add 180 ml. 5 *N* NaOH.
- (3) Make up the resulting solution to 1 l. with distilled water.
- (4) After thorough mixing, store the reagent in a brown glass container.

This reagent has been found to be quite stable for 2 weeks at room temperature. With refrigeration it may be kept several months.

#### *Hypochlorite solution:*

(1) Stir 50 gm. CaOCl in 600 ml. hot water (90 to 100° C.). Not all the material will dissolve.

(2) Add, with stirring, 270 ml. 20 per cent K<sub>2</sub>CO<sub>3</sub> solution, made up in previously boiled water. Mix and heat to 90° C.

(3) Filter through Whatman No. 1 paper into a 1-l. volumetric flask and adjust the volume to the mark with distilled water. Test a small amount for calcium by heating with some K<sub>2</sub>CO<sub>3</sub> solution.

(4) The solution should be clear and should contain 1.3 to 1.4 gm. free chlorine per 100 ml., which may then be tested.

(5) Place 2 ml. hypochlorite solution in a 20-ml. test tube.

(6) Add, in order, 10 ml. water, 2 ml. 5.0 per cent KI, and 1.0 ml. acetic acid.

\* Technicon Instruments Corp., Chauncey, N. Y.

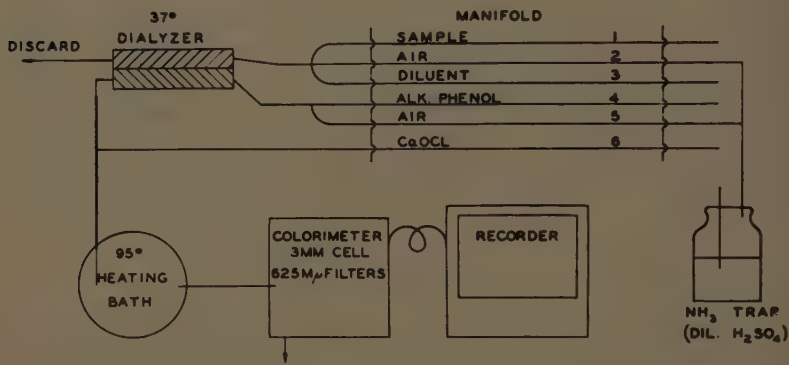
(7) Titrate this solution with 0.1 *N* sodium thiosulfate, using starch indicator. From 7.5 to 8.0 ml. sodium thiosulfate should be required.

This reagent is quite stable at room temperature when stored in a brown glass container.

*Diluent.* In order to maintain an alkaline medium on either side of the membrane in the dialyzer, a sodium carbonate solution is used as a diluent. A 1.0 per cent solution has been found satisfactory for most purposes.

### Instrumental

The determination is performed on the AutoAnalyzer, with the normal sequence of operations and components.



DONOR		RECIPIENT		CaOCL	
1 BLACK	0.32 ML. MIN.	4 GREEN	2.00 ML. MIN.	.6 YELLOW	0.6 ML. MIN.
2 YELLOW	1.20	5 YELLOW	1.20		
3 BLUE	1.60				
	3.12 TOTAL		3.20 TOTAL		

FIGURE 1. Ammonia determination manifold and flow chart.

*Sampling.* Thirty determinations per hour are performed by operating the sampling module at 60 per hour and alternating samples with cups of water. It is possible to run 40 samples per hour without the cups of water; however, resolution between samples is not good.

*Manifold.* Manifold components and tube sizes are given on the flow chart (FIGURE 1). The alkaline phenol is pumped directly, through ordinary Tygon manifold tubing. No adverse effect has been observed during the expected useful life of the tubing.

### Dialysis

A single 37° C. constant-temperature dialyzer is used. The alkaline phenol reagent serves as the recipient stream. Here again no ill effect on the dialysis membrane has been noted during the four weeks between routine membrane changes.



*Heating Bath*

The regular 95° C. heating bath is used.

*Colorimeter*

A 3-mm. flow cuvette is used primarily to provide superior resolution between samples. Absorption by the colored product is maximum at 610  $m\mu$ ; however, the peak is broad, and satisfactory results are obtained with the use of 625- $m\mu$  filters.

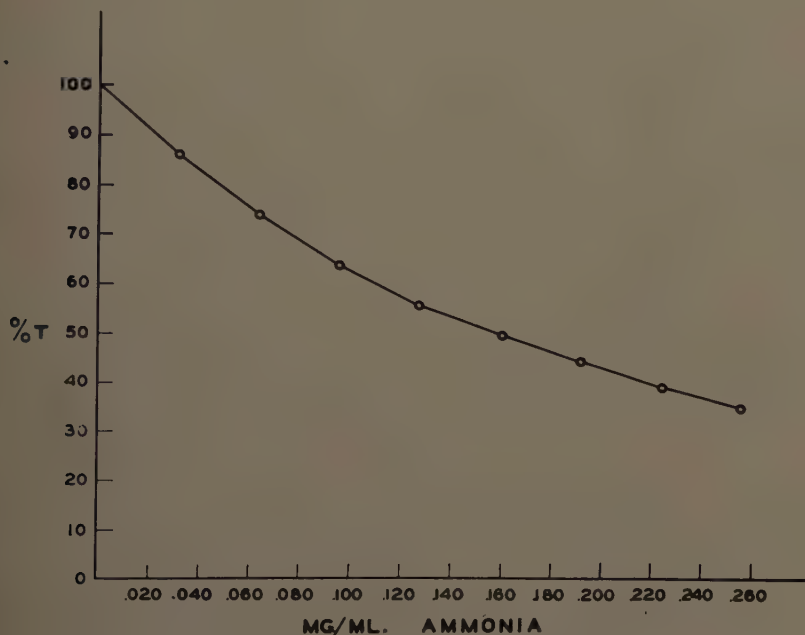


FIGURE 2. Typical ammonia standard curve.

## DISCUSSION

This method was developed primarily for the determination of ammonia in microbiological fermentation media. For this purpose a working range of from 0.010 to 0.260 mg. ammonia per ml. was established. A typical standard curve is shown in FIGURE 2.

It had been reported previously<sup>1</sup> that amino acids gave the same reaction as did ammonia, but to a lesser degree; this has not been our experience, under the conditions employed, as shown in TABLE 1. The amounts used of the amino acids cited are approximately equimolar to the amounts of ammonia added. On a weight basis they represent from 5 to 15 times the amount of ammonia present. The specificity of the alkaline phenol-hypochlorite reaction for ammonia was confirmed when the recovery of known amounts of ammonia was not altered in the presence of potential contaminants such as nitrogen

bases, dialyzable peptides of low molecular weight, and salts (*see* TABLES 2 and 3).

The reproducibility of the method has been well demonstrated by these studies. At an ammonia concentration of 0.100 mg./ml., the mean recovery

TABLE 1  
RECOVERY OF AMMONIA IN THE PRESENCE OF AMINO ACIDS

Acid	Recovery (%)
Glycine*	100
Tryptophan	100
Leucine	100
Cystine	100
Asparagine	100
Proline	102
Alanine	103
Tyrosine	100
Arginine	100
Phenylalanine	98
Ornithine	100
Diaminobutyric acid	100
Diaminopropionic acid	100
Citrulline	99
Mixture†	100

\* All acids were used in concentrations of 0.01 *M*; 0.1 mg.  $\text{NH}_4$  was added to each sample.

† Containing all acids listed above.

TABLE 2  
RECOVERY OF  $\text{NH}_4$  IN THE PRESENCE OF MISCELLANEOUS  
NITROGEN-CONTAINING COMPOUNDS

	$\text{NH}_4$ recovery (%)
Purine*	98
Guanine	98
Adenine	98
Adenosine	100
Prolylglycine	100
Prolylalanylglycine	100
Asparagylcysleiyglycine	100
Acetodiphenylalanine	100
Alanylglycylglycine	100
Adenylic acid	100

\* All compounds were 0.01 *M*; 0.1 mg.  $\text{NH}_4$  was added to each sample.

was found to be 100.0 per cent with a standard deviation of  $\pm 0.5$  per cent. Data were collected at two other ammonia levels, 0.06 and 0.200 mg./ml. The lower level showed a response of 100.0 per cent with a standard deviation of  $\pm 0.3$  per cent, and the high level yielded  $100.7 \pm 0.8$  per cent.

The determination of ammonia on very small quantities of material is a problem encountered in many experiments. The usual techniques require separation of the ammonia by aeration or diffusion. Neither of these has

proved completely satisfactory because (1) it is necessary to remove the ammonia by treatment with alkali, and such treatment causes certain types of compounds to decompose, releasing extraneous ammonia, and (2) in separating the free ammonia to an acidic recipient solution, the physical and mechanical problems involved are difficult to control or standardize.

Solutions containing as little as 0.5  $\mu\text{g./ml.}$  of ammonia may be successfully

TABLE 3  
RECOVERY OF  $\text{NH}_4$  IN THE PRESENCE OF SALTS

Salt	$\text{NH}_4$ recovery (%)
$\text{NaNO}_3^*$	100
$\text{NaC}_2\text{H}_3\text{O}_2$	98
$\text{NaHCO}_3$	100
$\text{NaCN}$	97
$\text{NaF}$	100
$\text{KI}$	103
$\text{K}_2\text{HPO}_4$	103
$\text{CaCl}_2$	98
$\text{FeCl}_2$	102
$\text{MgCl}_2$	100
$\text{MnSO}_4$	100
Mixture†	100
$\text{NaC}_4\text{H}_4\text{O}_6$	102
$\text{Na}_2\text{C}_2\text{O}_4$	102

\* All salts were 0.1 M; 0.1 mg.  $\text{NH}_4$  was added to each sample.

† Containing the 11 salts listed above it.

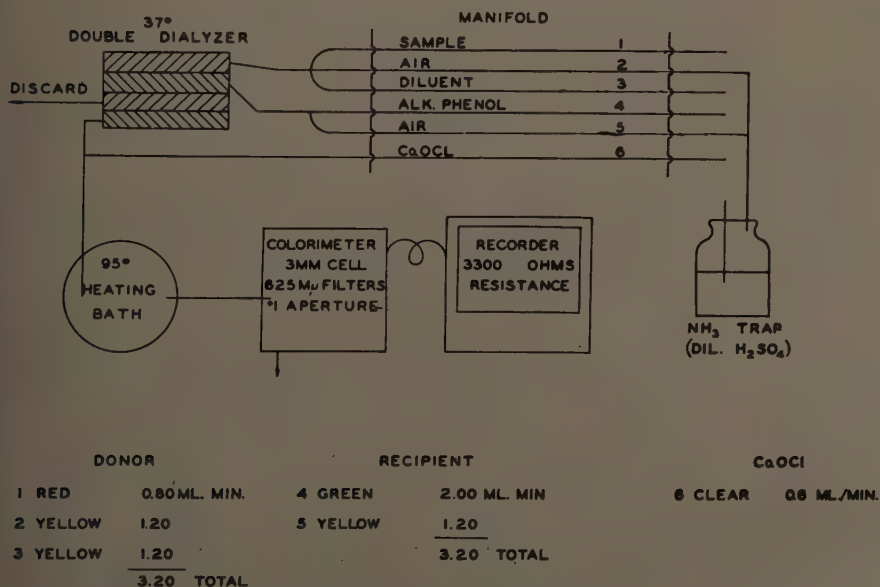


FIGURE 3. Micro-ammonia determination manifold and flow chart.

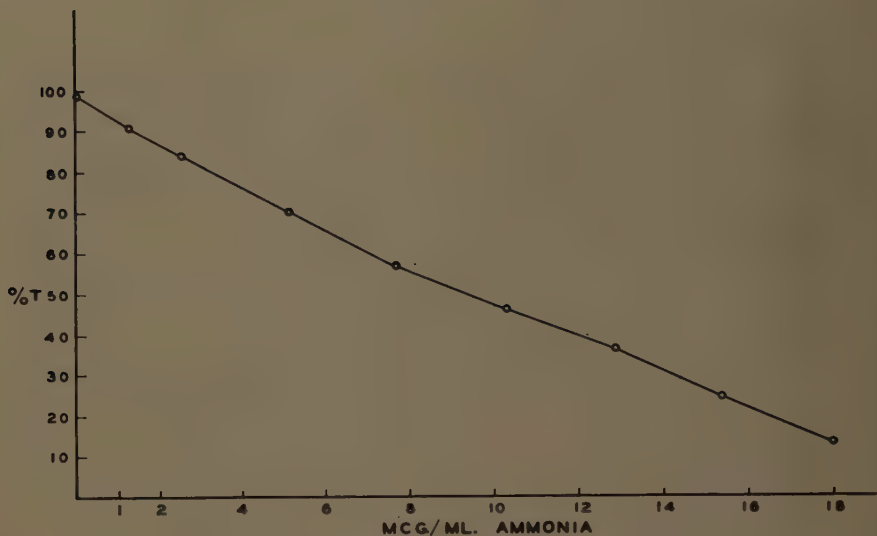


FIGURE 4. Typical micro-ammonia standard curve.

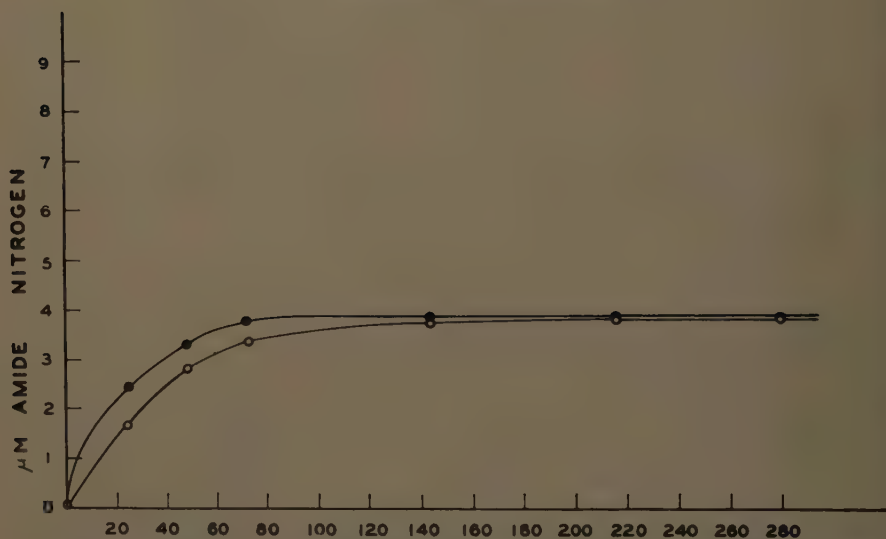


FIGURE 5. Glucagon, ○, and glutamine, ●, released from material incubated in concentrated HCl at 37° C. The units of the abscissa are hours of incubation.

TABLE 4  
FREE AMMONIA IN ANIMAL SERUM

Sample No.	Conway Cell (mg. %)	AutoAnalyzer (mg. %)
1	0.27	0.265
2	0.25	0.205
3	0.24	0.240
4	0.27	0.285
5	0.33	0.270
6	0.25	0.180
7	0.31	0.210
	0.275 ± 0.034	0.234 ± 0.031



analyzed on the AutoAnalyzer with the following minor modifications of the previous method:

The modified manifold components and tube sizes are given on the flow chart (FIGURE 3). The only significant difference is the increased sample size required. Dialysis is carried out with a 37° C. constant-temperature double dialyzer (two pairs of dialyzer plates connected in series). The technique of Taylor and Marsh (elsewhere in this monograph) for expanding the range of the recorder by adding resistance to the negative side of the reference photocell is utilized, and 3300 ohms resistance is added. A No. 1 aperture is necessary in the colorimeter.

With these modifications, a standard curve for ammonia was established from 1.0 to 18.0  $\mu\text{g./ml.}$ , as is shown in FIGURE 4. This method has been used successfully in amide nitrogen studies to determine the ammonia released by  $\mu\text{mole}$  quantities of proteins, as described by Rees;<sup>2</sup> in these studies the test material was incubated at 37° C. in concentrated HCl for a sufficient length of time to liberate the amide nitrogen as free ammonium ions. The results of such a study are shown in FIGURE 5.

The complete amide nitrogen study was performed in duplicate with 1  $\mu\text{mole}$  of the protein glucagon (mol. wt. 3500, 4.0 residues of amide nitrogen per mole). Four micromoles of glutamine were treated in a similar manner, to be used as controls. Dilutions were made so that 1  $\mu\text{g.}$  of ammonia corresponded to 1 residue of amide nitrogen. In the controls, 3.9 residues were recovered from a possible 4.0. The test material glucagon yielded 3.85 residues of the 4.0 present. In previous work by other techniques, acceptable results from comparable determinations have required at least 20 times as much material.

This micromethod has been used to determine also the free ammonia content of blood serum in laboratory animals. No extensive effort has been made to correlate the results obtained with those of other methods. However, in a limited number of comparisons with the Conway microdiffusion method, it was found that the AutoAnalyzer results were almost always somewhat lower than those of the Conway method (TABLE 4). Since the values so obtained were from unadulterated samples, it is possible that they represent the true serum ammonia values. Furthermore, it should be pointed out that the differences, although they seem quite marked, are actually rather minor, since the normal range for serum ammonia is from 0.1 to 1.1 mg. per cent.<sup>3</sup>

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# CONTINUOUS AUTOMATIC CHEMICAL ANALYSIS OF NITRATE IN THE PRESENCE OF AMMONIA AND UREA

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The determination of nitrate or, more specifically, ammonium nitrate, is very important in the fertilizer industry. The official methods as described by the Association of Official Agricultural Chemists<sup>1</sup> are relatively cumbersome and time-consuming, owing to the interferences of urea and/or ammonia. In the absence of these interferences, nitrate may be determined colorimetrically with the use of phenol disulfonic acid,<sup>2</sup> diphenylamine,<sup>3</sup> or any of several other compounds.<sup>4</sup>

With the methods commonly employed, the interferences due to ammonia and urea necessitate many manipulations in a routine analysis. Hours are required for the digestion with urease, reduction with Devarda's metal, distillation, and titration in each analysis. Obviously, if analysis is to be made automatic and continuous, the lengthy treatments must be eliminated, while at the same time a color reaction suitable for colorimetry must be found. It is the purpose of this paper to describe the adaptation of the Technicon Auto-Analyzer\* for this type of analysis, and to present a brief description of the instrumental system.

The Technicon AutoAnalyzer is a modular-type instrumental system.<sup>5</sup> The principal modules used in this work were the proportioning pump, the 37° C. constant-temperature dialyzer, the 95° C. constant-temperature heating bath, the continuous-flow colorimeter, and the strip chart recorder.

The proportioning pump is the heart of the system. It consists of two parallel stainless steel roller chains with spaced thwarts that bear continuously against a spring-loaded pump platen. On this platen is placed a set of flexible Tygon tubes whose different lumens determine the rate of flow through each. As the chain advances across the platen, the rollers move across the tubes, providing an advancing wave of compression that forces the fluids through them in the exact proportions required by the particular test. Glass fittings are provided for the manifoldings of the various tests, and mixing is performed by repeated inversion as the fluids pass through a series of glass helices. All of the fluids in the system are proportioned simultaneously, manifolded, segmented by air, and mixed at a rate dependent upon the diameter of the tubes.

The constant-temperature dialyzer is employed wherever purification of the original sample is required, as with samples containing suspended matter. In addition, where relatively high concentrations are encountered, it serves as a dilution factor. The dialyzer consists of accurately milled grooves in two Lucite plates, one being the mirror image of the other. Suitable clamping plates are provided so that, when the Lucite plates are assembled with a sheet of semipermeable membrane between them, two identical tubular passages are

\* Technicon Instruments Corp., Chauncey, N. Y.

created. The sample stream is passed on one side of the membrane and the recipient stream on the other.

To ensure constant rates of dialysis, the dialyzer assembly is immersed in a constant-temperature bath. Since the amount of material dialyzed depends on the area of the dialyzing membrane, the time of exposure to the membrane, the temperature at which dialysis takes place, and the concentration, it is evident that the first three variables must be maintained constant in order to measure the fourth. Area and time are controlled by the dimensions of the system and the proportioning pump, while temperature is maintained at  $37 \pm 0.1^\circ \text{C}$ .

The heating bath consists of a double-walled vessel electrically heated and regulated to  $95 \pm 0.1^\circ \text{C}$ . Within this vessel is placed a heat exchange coil generally 40 feet long with a volume of approximately 27 ml. The duration of heating time depends, of course, on the volume of the coil and the pumping rate through the coil.

The dual-beam colorimeter requires two separate photoelectric cells: one for reference and the other for measuring. The main feature of the colorimeter is the flow cuvette. In this cuvette, the air-segmented reaction stream is received in a premixing chamber that serves also to remove the air segment. Via continuous overflow, the reacted sample flows from the premixer into the viewing chamber. Interference filters of 10  $\text{m}\mu$  half-band width are employed to provide a source of monochromatic light with which to scan the reacted stream. The change in absorbance is then continuously measured and recorded. The recorder is of the usual industrial type, modified for ratio recording in conjunction with the dual-beam colorimeter.

The basis of the method employed was given in a paper by Englebrecht and McCoy<sup>6</sup> describing an oxidimetric reaction of nitrate and ferrous sulfate followed by a titration of the excess ferrous sulfate by potassium permanganate. Neither urea nor ammonia interfere. To the sample is added first a sulfuric acid solution of ferrous sulfate and sodium chloride, and then concentrated sulfuric acid. The dark brown solution is boiled gently for three to five minutes until a permanent orange color is obtained. At this point the reaction mixture is diluted and titrated with standard permanganate. With urea present, sodium chloride is added to the sample aliquot before the ferrous sulfate. The authors state that without the sodium chloride low nitrate values were obtained, probably owing to the formation of urea nitrate. With the addition of sodium chloride, and under the acidic conditions of the analysis, formation of urea hydrochloride prevails over formation of the nitrate.

On the basis of this description, manifold A was devised (FIGURE 1). The sample (0.32 ml./min.), air (0.8 ml./min.), and 1.2 ml./min. of 15 per cent sodium chloride were mixed and sent into the dialyzer. The recipient stream consisted of 2.0 ml. of 1 *N* ferrous sulfate reagent mixed, after dialysis, with 2.5 ml. of 50 per cent v/v sulfuric acid, the combined streams being heated at  $95^\circ \text{C}$ . in the heating bath. To the heated stream was added 1.2 ml./min. of 0.5 *N* potassium permanganate; these streams were mixed and sent into the flow cell of the colorimeter, where absorbance was read at 575  $\text{m}\mu$ . It must be

noted that this manifold follows only generally the scheme outlined by Englebrecht and McCoy, for several reasons:

(1) The choices of reagent strengths and volumes to be used on the Auto-Analyzer are relatively limited as compared to the wide choice available with a manual technique.

(2) Strict completion of the reactions is not necessary, since all reactions are always carried out to the same degree at the same level of concentration.

(3) Strict standardization of reagents is not needed, since an instrumental standardization is carried out periodically.

Several standards of ammonium nitrate run in manifold A gave such extremely jagged deflections that it was impossible to estimate their values. Improving the mixing, segmenting the sulfuric acid stream, and increasing the

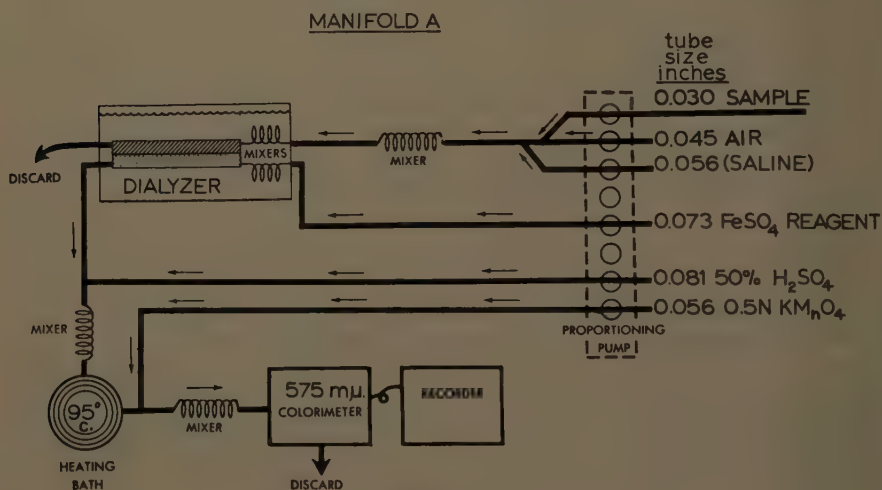


FIGURE 1

saline concentration smoothed out the results considerably. A double dialyzer was employed to increase sensitivity. The final manifold is shown in FIGURE 2. Here it is seen that a segmented stream of 0.32 ml./min. of sample, 1.2 ml./min. of 15 per cent sodium chloride, and 0.8 ml./min. of air is mixed and sent into the sample side of a double dialyzer. The recipient stream is the ferrous sulfate reagent pumped at 2.0 ml./min.; upon emergence from the dialyzer, it is joined by an air-segmented stream of 50 per cent v/v sulfuric acid. The acid and air are pumped at 2.5 and 0.8 ml./min., respectively. After mixing, the combined streams are heated at 95° C. for a few minutes to allow oxidation to take place. The stream then joins 1.2 ml./min. of 0.5 N potassium permanganate for oxidation of the excess ferrous sulfate, which occurs during passage through the mixing and the time delay coils. The nitrate concentration then is directly proportional to the permanganate color of the final reaction stream. It appears that, owing to the incomplete reaction between the nitrate and ferrous ion, the color due to formation of  $\text{FeNO}_2$ , an addition compound of



nitric oxide and ferrous sulfate, also contributes to the extent of deflection.<sup>7</sup> This can be tolerated since both colors are indicative of nitrate concentration.

The next phase of experimental evaluation was testing the analytical system for reproducibility and for the effect of varying concentrations of ammonia and urea. The ammonium nitrate in the samples tested ranged from 0.5 to 2.0 weight per cent. A number of standards were prepared containing varying amounts of ammonia and/or urea, the concentrations being chosen to represent the approximate ranges usually met with in the fertilizer industry, at a dilu-

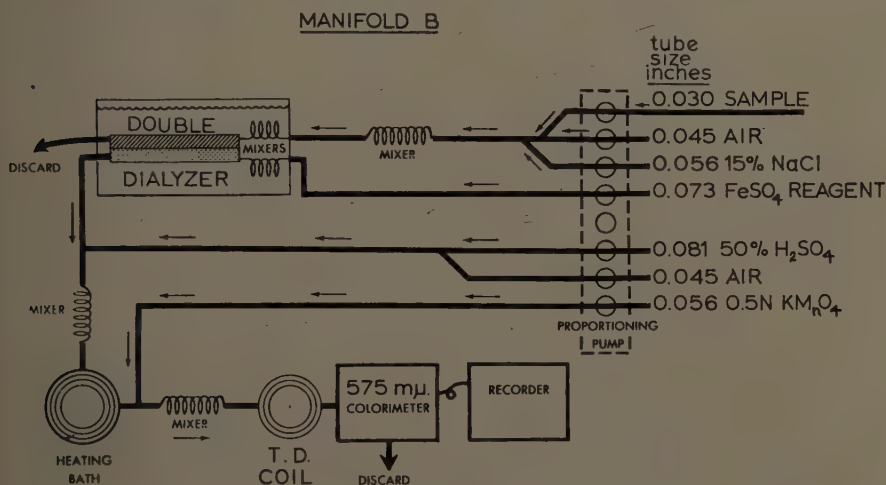


FIGURE 2

TABLE 1

Nitrate concentration (%)	Mean	Standard deviation	Coefficient of variation
2.00	1.99	0.063	3.2
1.50	1.49	0.050	3.4
1.00	1.00	0.027	2.7
0.50	0.50	0.017	3.4

tion of 1:50. These concentrations were 0.1, 0.3, 0.5, 0.7 weight per cent urea and 0.2, 0.4, 0.6, 0.8 weight per cent ammonia.

After calibration of the analytical system with pure ammonium nitrate standards of 0.5, 1.0, 1.5, 2.0 weight per cent, the interference-containing standards were run for comparison. Twenty-five samples were run at each nitrate level, and the results are shown in TABLE 1. The means, standard deviations, and coefficients of variation are given for the indicated levels of nitrate concentration for complete ranges of concentrations of ammonia and urea contaminants. These results indicate a good degree of validity and reproducibility. An important conclusion to be drawn from the results is the lack of interference due to the presence of ammonia and/or urea. Here, then, is a

basic method complying with all the aforementioned requirements as to reproducibility, noninterference from contaminants, and so forth.

One rather important aspect of the procedure requires further development. This is the matter of increasing the resolution to the point where nitrate concentrations of 50 per cent and greater may be read to the desired degree of resolution. Work on this aspect of the problem is now being carried out. At present, the following approach is being taken.

Increasing the temperature of the heating bath to 110 or 115° C. will increase the extent of oxidation of the ferrous ion by the nitrate and thus provide greater differentiation between any two given concentrations. The reagent system will be sensitized by dilution of the ferrous sulfate and potassium permanganate, thus permitting a given nitrate concentration to oxidize a correspondingly greater proportion of the ferrous ion. It must be emphasized at this point that the laws of colorimetry being what they are, as resolution is increased, range is correspondingly decreased; consequently, when the final analytical system has been evolved, the over-all range will be limited to a 5 or 10 per cent difference in transmission. This, however, should be entirely satisfactory, since by suitable manipulation of reagents this 5 or 10 per cent range may be taken anywhere over the 50 to 100 per cent over-all range.

### *Summary*

A system capable of continuously and automatically analyzing for nitrate in the presence of ammonia and urea has been developed for a low range of nitrate concentrations. Work is now being directed toward obtaining the same degree of resolution in the very high concentrations.

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## INDUSTRIAL APPLICATIONS OF THE TITRILOG

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The problem of analyzing for sulfur compounds, especially in trace quantities, has always been very important in a large number of industries and is rapidly increasing in scope with the continuous strides industry is making in processing materials, many of which contain sulfur compounds. Trace quantities of sulfur are often detrimental to efficient and economical operation. Likewise, the emphasis on safety measures such as the odorization of nonodorous natural gas with certain sulfur compounds for domestic use and the monitoring of atmospheres for dangerous concentrations of toxic sulfur compounds has enlarged the problem of sulfur compound analysis. For example, sulfur dioxide at 400 to 500 ppm is immediately dangerous to life, and 50 to 100 ppm for 30 to 60 min. is the maximum tolerable level for humans.

With regard to toxic effects, hydrogen sulfide in high concentration can cause immediate death. Special gas masks must be worn when hydrogen sulfide is present in the concentration range of 20,000 ppm. The conventional chemical methods of analysis of such atmospheres present inadequacies, not only because of the time required, but also because of inaccuracies in the lower measurement levels and the ever-present doubts resulting from spot checks.

It is, therefore, the purpose of this paper to discuss the operation of an automatic and continuously recording titrator commercially known as the Titrilog,\* and to describe its applications in some specific industrial sulfur problems.

### *The Titrilog*

FIGURE 1 shows the 26-103B Portable Titrilog. This chemical-electronic instrument has been designed continuously to record concentrations of oxidizable sulfur compounds such as hydrogen sulfide, sulfur dioxide, mercaptans, thiophene, and organic sulfides and disulfides in gas streams or atmospheres. The range of the instrument can be varied over wide limits by varying the rate of sample introduction. At maximum sensitivity the Titrilog is capable of recording concentrations as low as 0.1 ppm by volume, or 0.005 grains/100 cu. ft. A very interesting feature of the instrument is its speed of response, which allows changes in concentration to be recorded within 20 to 30 sec.

The measurement of the sulfur compounds is accomplished by a titration with bromine, as shown in FIGURE 2. The bromine is generated electrolytically in a solution in which the sulfur compounds are absorbed from the gas stream. The feedback amplifying system controls the bromine-generating current so that the net rate of bromine generation is at all times equivalent to the rates of absorption of the sulfur compounds. A recording milliammeter records the generating current. The net current is proportional to the sulfur compound concentration in the incoming gas stream. The feedback amplifying system

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is composed of a DC amplifier, a DC reference voltage, and the titration cell. The recording milliammeter measures the amplifier output current.

There are two sources of voltage in the input circuit. The first is a reference voltage from a stable source such as a battery. The second input voltage is that of the bromine-sensitive electrode in conjunction with a reference electrode in the titration cell. These two voltages are opposed. The net voltage from these two sources acts as a control on the amplifier output which is the bromine-generating current. The reference voltage is set to equal the voltage of the bromine-sensitive electrode at a fixed bromine concentration. When the two are equal, the potentiometric balance point has been established, and no signal is fed to the amplifier. If the bromine concentration falls below the desired



FIGURE 1

level, the sensor voltage will fall below the stable reference voltage, producing an input voltage to the amplifier. The amplifier responds to increase the bromine-generating current to the titration cell. Thus, the bromine concentration is increased and, consequently, the voltage from the bromine-sensitive electrode increases until the input approaches zero. In this manner, the feedback system acts automatically to maintain a set concentration of bromine in the titration cell.

Because of the continuous flow of gas through the titration cell, a small amount of bromine is always being swept out. Therefore, a small signal is sent continuously to the amplifier, and the generating current never falls to zero. When the amplifier is set at high gain, the output will reflect extremely small signals resulting from very small changes in bromine concentration.

When a gas that will react with the bromine enters the titration cell, the bromine concentration is reduced, and the voltage from the bromine-sensitive



electrode drops momentarily so that a signal is sent to the amplifier. The amplifier responds immediately, increasing the bromine-generating current so that now the bromine requirements of the reaction are satisfied and the desired residual bromine concentration corresponding to the reference voltage is maintained. Thus the reaction is carried out continuously at a point approaching

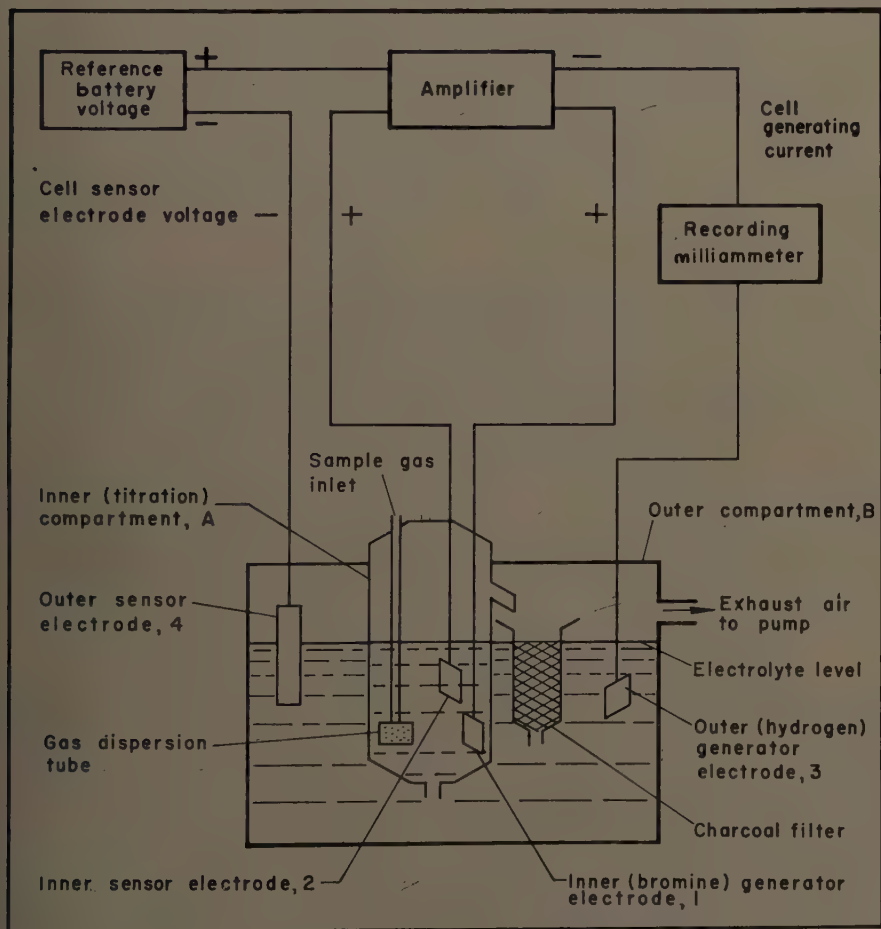


FIGURE 2. Titrilog cell and amplifier.

the equivalence of the two reactants. The recording milliammeter in the amplifier output circuit records the bromine-generating current. Since, according to Faraday's law, the quantity of bromine generated is a function of the current, the record of the current is an indication of the extent of titration. In an oxidation-reduction reaction, the voltage developed at the sensing electrode is a logarithmic function of the concentration. Therefore, as the point of equivalence is approached, the change in voltage is large. Utilization of the feedback principle in a high-gain amplifier allows the reaction to take place near

the point of equivalence, and the resulting instrument is extremely sensitive and rapid in response to changes in sulfur compound concentrations.

The titration cell (FIGURE 2) is a glass vessel containing an electrolyte of dilute sulfuric acid, 7.5 *N*, and potassium bromide, 0.1 *N*, from which the bromine is generated. The concentrations can be varied over wide limits. The titration reaction takes place in the inner cell chamber A, where the sample gas is introduced continuously and the sulfur compounds are absorbed. Bromine is generated from Electrode 1. Electrode 2 in the same compartment is a platinum electrode responsive to changes in bromine concentration. The outer chamber B acts as a reservoir for the electrolyte and contains Electrodes 3 and 4, which complete the electric circuit. Electrode 3 completes the electrolysis reaction, generating hydrogen that is vented, taking no part in the titration reaction. Electrode 4 is a calomel half-cell type of reference electrode against which the voltage of the bromine-sensitive Electrode 2 is developed.

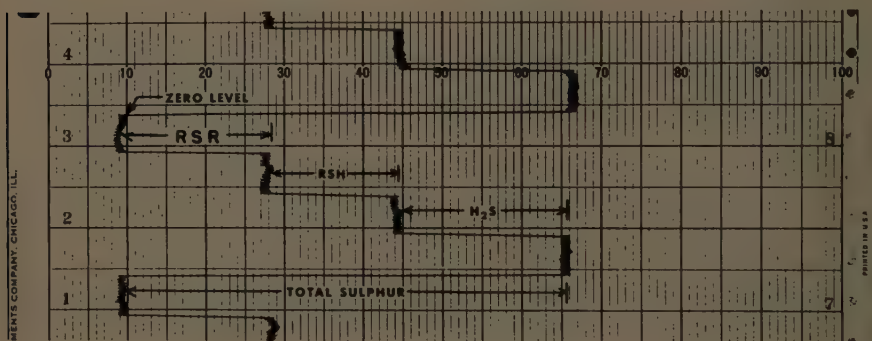


FIGURE 3. Typical Titrilog record.

Instead of the conventional mercurous chloride, mercurous bromide is used. The electrode is in contact with the electrolyte and is therefore 0.1 *N* with respect to potassium bromide. The reference voltage obtained from this electrode, measured against that of the sensor electrode at zero bromine concentration, is of the order of 750 mv. The balancing reference voltage, from a dry-cell battery, is connected in the circuit to oppose the voltage between the bromine-sensitive Electrode 2 and the calomel-type reference Electrode 4. As previously mentioned, the voltage of the bromine-sensitive electrode will tend, by design, at all times to approach that of the balancing reference voltage. Prior to the introduction of a sample gas containing a reactant, filtered air is passed through the cell, and the balancing reference voltage is adjusted for some desired bromine concentration. Because the air passing through the cell continuously carries out a very small amount of bromine, the generating current never drops to zero, and the amplifier operates continuously to equalize the rate of generation to the rate of loss. This steady rate of generation is called the zero level, and in practice is set for a very low bromine concentration. FIGURE 3 shows a typical Titrilog record. Note the low bromine concentration used as a zero level.

There are two types of Titrilog sampling systems. The first is the vacuum sampling system, used where there is no pressure line (stream) to which the Titrilog can be connected. The second is the pressure-type sampling system, used with a pressure line. These two systems are shown in FIGURES 4 and 5.

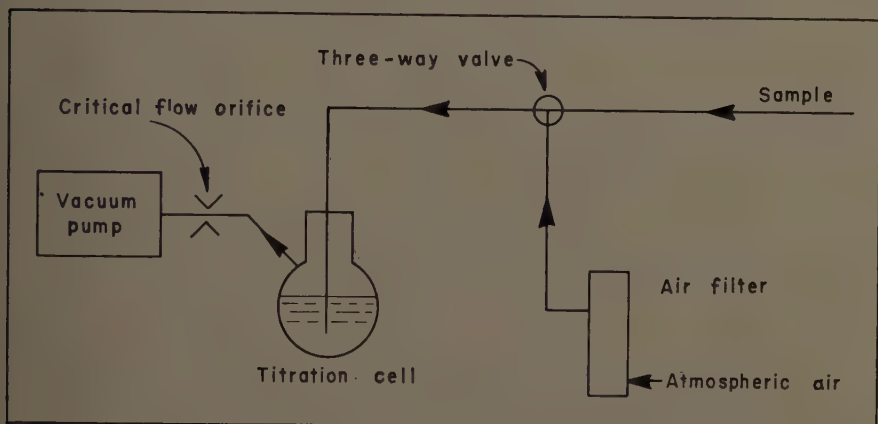


FIGURE 4. Titrilog vacuum-sampling system.

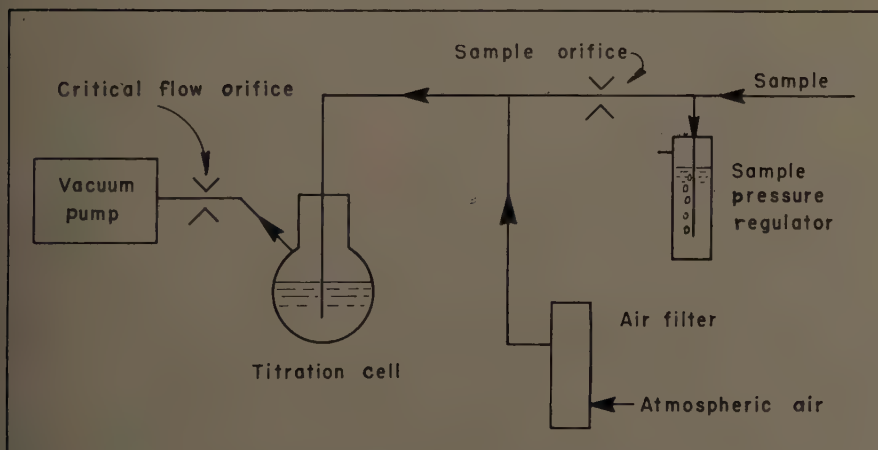


FIGURE 5. Titrilog pressure-sampling system.

### *Industrial Applications*

The industrial applications of the Titrilog fall generally into four main categories: personnel protection systems, air pollution studies, catalyst protection systems, and gas odorization control.

For personnel protection, the Titrilog will be found measuring concentrations of such gases as hydrogen sulfide and sulfur dioxide. Generally, a concentration of 10 ppm is considered by toxicologists to be the maximum allowable for

either gas. The Titrilog is used also to actuate alarms or turn on warning lights and/or fans when the concentration of the gas approaches a level dangerous to personnel.

The Titrilog is used in such places as isolated gas monitoring stations in Canada, which may be visited only once or twice a week. If, during one of these unattended periods, the gas concentration exceeds a given level in the station, the Titrilog actuates blower-type fans and operates a warning light which can be seen from the outside of the gas monitoring station. The station is never entered by personnel when the warning light is on.

At the Sheritt-Gordon Mines,\* a process for treating slurried ores utilizes highly toxic hydrogen sulfide. A Titrilog has been installed to actuate a warning alarm if the concentration exceeds 10 ppm in the work areas.

The Titrilog has been mounted on a truck and used to check gas concentration "pockets" in areas around natural gas plants.

An application of increasing importance is the study of air pollution. Sulfur dioxide is one of the principal offending agents and is generally an index of the level of pollution. In some instances, hydrogen sulfide or mercaptans are also of interest. Since the Titrilog is capable of recording trace quantities of these compounds, it is being used effectively in these studies. The concentration range generally encountered is of the order of less than 0.1 ppm to 3 or 4 ppm. In this type of application, the instrument can record only the total amount of all titratable substances; no successful method of selective separation has yet been devised. Titrilog records are being used in surveys of areas for sources of pollutants, in correlation with meteorological data. Such correlated data seem to indicate that damage by pollutants may be due not to low average concentrations but to short periods of peak concentrations.

An interesting recent application of the Titrilog, initiated by Fred Thomas of T. V. A., has been the recording of  $\text{SO}_2$  concentrations at upper levels in the vicinity of industrial stacks, from a cruising helicopter. Owing to the Titrilog's rapid response, complete concentration profiles are obtained while the helicopter cruises at 30 mph. No other instrument is sufficiently portable, rugged, or quick in response for this application.<sup>1</sup>

More than twenty people died and hundreds were made ill during the 1948 smog incident at Donora, Pa. United States Steel's Donora Zinc Works was a natural target for blame. Subsequently, this company used three Titrilogs to monitor the atmosphere in the Donora area for purposes of study and to insure against repetition of such a disaster.

D. F. Adams, of Washington State College, uses the Titrilog in a mobile unit to check  $\text{SO}_2$  concentration at thirty different locations in an eight-hour shift.<sup>2</sup> We now have developed an accessory for the Titrilog that increases its sensitivity to  $\text{SO}_2$  to the parts-per-billion range.

The Titrilog is used in conjunction with catalyst protection systems. Quite often, extremely small traces of sulfur compounds are detrimental to the efficiency of catalysts. In a plant synthesizing ammonia from natural gas, it was found that the hydrogen-nitrogen reaction catalyst frequently lost efficiency.

\* Fort Saskatchewan, Alberta, Canada.



Continuous monitoring of this stream indicated a sulfur concentration in the order of 4 ppm. The Titrilog was used to reach the source of this sulfur, and corrections were made to eliminate it.

Trace quantities of sulfur compounds could reduce greatly the activity of the expensive catalyst used at Phillips Chemical Company's Adams Terminal Ammonia Plant.\* A Titrilog continuously monitors the treated natural gas feed stream to insure against any contamination.

At Dow Chemical Company's ammonia plant at Velasco, Texas, the Titrilog traced sulfur to an obscure source: tidal water contamination of process water.

The odorization of natural gas is complicated where the gas contains variable concentrations of natural odorous compounds. If a commercial odorant is added to the gas at a fixed concentration, overodorization will occur at times with resultant increase in leak complaints, whereas insufficient odorization will occur when natural odorant is low. Where natural gas contains appreciable quantities of sulfur compounds, some hydrogen sulfide is usually present. The gas therefore is processed to reduce the concentration of hydrogen sulfide to tolerable limits.

Hence it is important to have full information on the efficiency of  $H_2S$  removal and, where plant failure does occur with high-level  $H_2S$  contamination resulting, it is important to know the  $H_2S$  concentration and the duration of the period of contamination. Spot sampling to obtain this type of information is costly if carried out with sufficient frequency to give very complete knowledge, and it often fails entirely to detect high concentrations of short duration. FIGURE 6 shows a typical Titrilog record for natural gas. The Titrilog was first applied to this problem on the Texas-California pipeline during the early period of its operation, when the content of  $H_2S$  and mercaptans in the gas was under study (see FIGURE 7).

This application provides basic field data on several characteristics of performance of the Titrilog under widely different field conditions. Among these are continuity of service, maintenance service requirements, and stability of operation. The Titrilog has operated continuously and satisfactorily over a wide range of environmental conditions.

The Public Service Company of Northern Illinois uses (1) a portable Titrilog to check odorization at many points in a complex system and to study diffusion of specific odorants in their system, and (2) a cabinet-model Titrilog to monitor the odorant and hydrogen sulfide content of the gas purchased from a transmission company.

Metropolitan Utilities in Omaha had to service 1000 leak complaints of overodorization in one day, before installation of a Titrilog. The Titrilog, equipped with a high-concentration alarm, has prevented recurrence of such trouble.

The Titrilog is being used more and more as a process-monitoring instrument. For example, at Dow Chemical Company's bromine-from-sea water pilot plant at Freeport, Texas, a small excess (40 ppm) of sulfur dioxide is required in a process stream. In this case, the Titrilog is used actually to control the  $SO_2$  feed rate.

\* Houston, Texas.

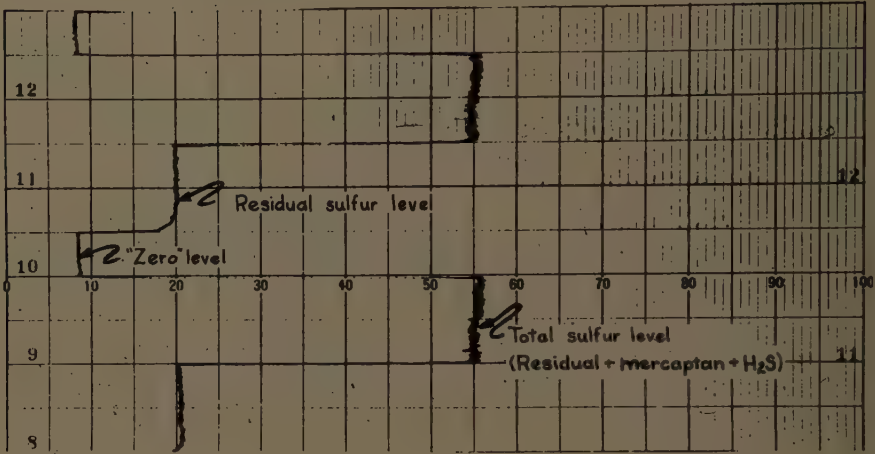


FIGURE 6. Typical Titrilog record for operation on natural gas.

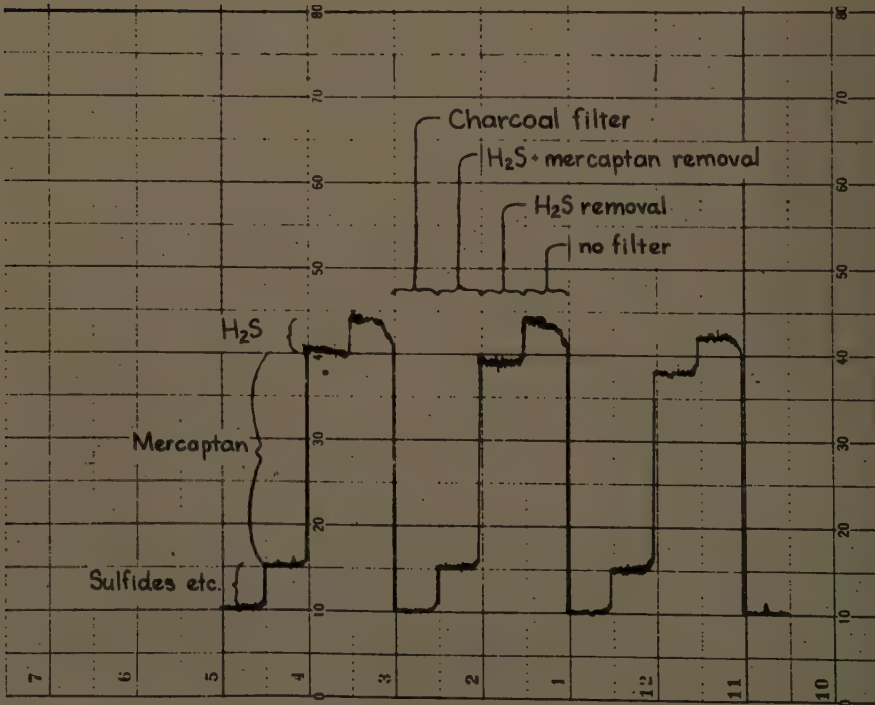


FIGURE 7. Titrilog analysis of Texas natural gas.

The Titrilog in a unique application may be seen on television when the American Chicle Company advertises Clorets: it is the Titrilog that senses the odorous sulfur compounds in onion oil.

### *Conclusions*

The Titrilog has been applied to a wide range of field problems involving the measurement of sulfur concentrations in gas streams. Practical operation of the instrument under field conditions has extended over a period of more than ten years. Installations in isolated locations have operated satisfactorily for extended periods under widely variable conditions of temperature and humidity and in locations where severe vibration is present.

In all cases, recording of the concentration of sulfur compounds by the Titrilog has been continuous and automatic. The Titrilog has been designed to meet the needs of pipeline monitoring applications where the concentrations of naturally occurring as well as commercial sulfur compounds are to be recorded. Modifications have been added to provide actuation of alarm and control elements to meet the requirements of such applications as process control. The portable Titrilog is designed for general laboratory or field use.

The field applications discussed in this paper are representative of the varied problems to be met in the gas industry and those related to it. The data presented show the Titrilog to be well adapted to the needs of these industries

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# THE SIMULTANEOUS DETERMINATION OF SAMPLE AND BLANK BY MEANS OF DUAL AND DIFFERENTIAL COLORIMETRY IN THE CONTINUOUS ANALYSIS OF PENICILLIN IN FERMENTATION MEDIA

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As shown in preceding papers, modern analytical instrumentation has been used to advantage in the automation of many industrial chemical processes. In the fermentation industries, advances have been made recently in the automation of analytical methods routinely used in the production of antibiotics. These advances have been made possible primarily by a recently developed automatic system for colorimetric chemical analysis via a continuous flow system. This instrumental system is known as the AutoAnalyzer.<sup>1†</sup> Details of the instrumentation have been described adequately.<sup>1,2</sup> Since the standard determination of penicillin potency in fermentation broth requires numerous and time-consuming repetitive analyses, our first attempt at automation was to adapt the iodometric method for determining penicillin to this new system.

## *Methods and Instrumentation*

The diagram of the manifold for the iodometric method is seen in FIGURE 1. The sample is segmented by air and then mixed in the mixing coils with a stream containing 750 U. penicillinase A‡ per milliliter. This mixture is passed into a 5-min. delay coil at room temperature for adequate inactivation of penicillin to penicilloic acid.

The inactivated stream then is passed into the dialyzer and dialyzed against a segmented stream of buffer consisting of 1 *N* monosodium phosphate adjusted to pH 6.5. A portion of the penicilloic acid is diffused into the buffer stream. The diffusate, on emerging from the dialyzer, is mixed in a mixing coil with a stream of 0.01 *N* buffered iodine and passed into a 5-min. time-delay coil, at room temperature, to effect the iodination of penicilloic acid. The stream emerging from this time-delay coil passes into the colorimeter, where the optical density is recorded at 420 m $\mu$ .

This method of calculating antibiotic potency requires that a blank be determined with every sample. This is accomplished by substituting distilled water for penicillinase in the same manifold. Potencies are calculated by converting the recorded optical densities of blank and unknown into potencies from a standard curve and subtracting the blank from the unknown.

The earliest trials with this method gave reasonably satisfactory results in most cases; however, as broader concentration ranges of widely varying fermentation media were encountered, it became evident that the method had a

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number of limitations. Valid analysis of many fermentation media was confined within rather narrow limits; furthermore, with some fermentation media the response of samples serially diluted with water was nonlinear, and lack of parallelism among blanks, unknowns and standards was observed. The reasons for these phenomena are not entirely clear. However, one contributing factor is that the rate of dialysis of penicilloic acid is not linear with concentration. In addition, the effects of other broth constituents on all steps in the method varied from medium to medium and could not be assessed or controlled easily. Attempts to use the method omitting dialysis were unsuccessful, owing to gross interference by broth constituents.

Another method for colorimetric analysis of penicillin is based on its reaction with hydroxylamine and ferric ion to give a colored complex.<sup>3</sup> This was inves-

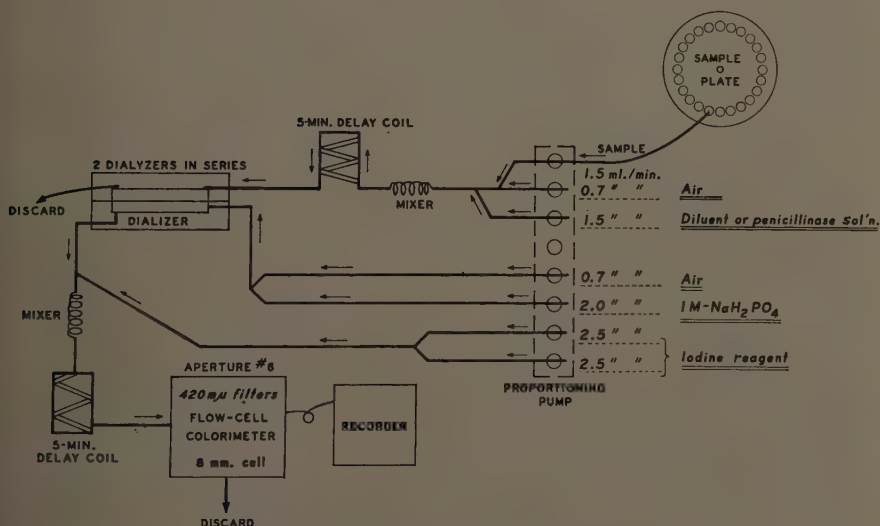


FIGURE 1. Flow diagram of iodometric penicillin method.

tigated for adaptation on the AutoAnalyzer. The continuous dialysis step was included and, as before, it resulted in a curvilinear response and a limited range of valid analysis. Hence it was decided to study this method omitting dialysis.<sup>4</sup> This would require that the fermentation samples be centrifuged before introduction into the sampling unit. The flow diagram is shown in FIGURE 2.

As in the iodometric method, for each estimate of penicillin potency a blank and an unknown determination are needed. In operation, the unknown is picked up from the sampling unit, segmented by air, and diluted with a stream of distilled water. The mixture is pumped through a standard mixing coil and then through a 5-min. time-delay coil. After this period it is joined by an unsegmented stream of hydroxylamine reagent and passed through a second mixing coil. After mixing, it is met by a stream of ferric ammonium sulfate reagent, mixed again, and passed through a 3-min. time-delay coil for color development. The colored stream then enters the flow cell of the colorimeter,

where the optical density is measured at  $480\text{ m}\mu$  and recorded. The flow system for the blank determination is exactly the same except that an aqueous penicillinase solution is substituted for distilled water. In this method, all samples are separated by distilled water samples to decontaminate the system adequately and permit return to the base line.

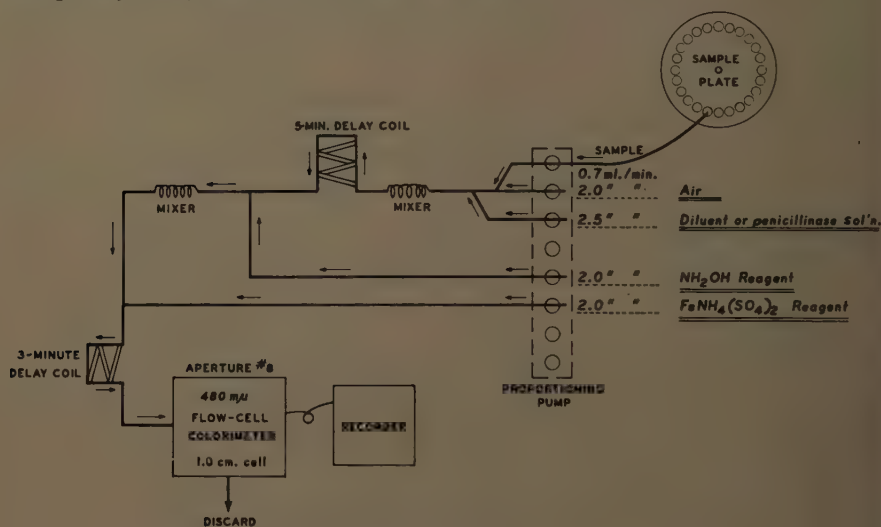


FIGURE 2. Flow diagram of penicillin hydroxylamine method.

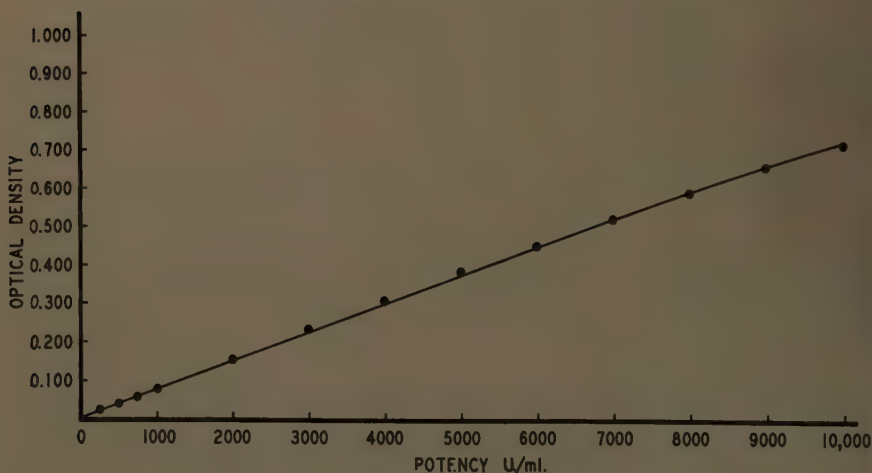


FIGURE 3. Aqueous penicillin standardization.

A typical standard curve obtained with aqueous penicillin standards (FIGURE 3) gives an almost linear response. Experience has shown that it is reproducible from day to day with an over-all error of 1 per cent or less. Furthermore, no nonlinearity or lack of parallel response of unknowns, blanks, or standards has been encountered. For these reasons this method is now used to determine penicillin content in all phases of manufacture and formulations.

Nevertheless, in the determination of penicillin by either method, a minimum blank value almost always is obtained for broth and process samples, and hence it is required that a blank analysis of the sample be determined either prior to or after the actual total analysis of the sample. To expedite the analysis of unknowns it was necessary to use two AutoAnalyzer systems wherein blank and sample were determined at the same time. This necessi-

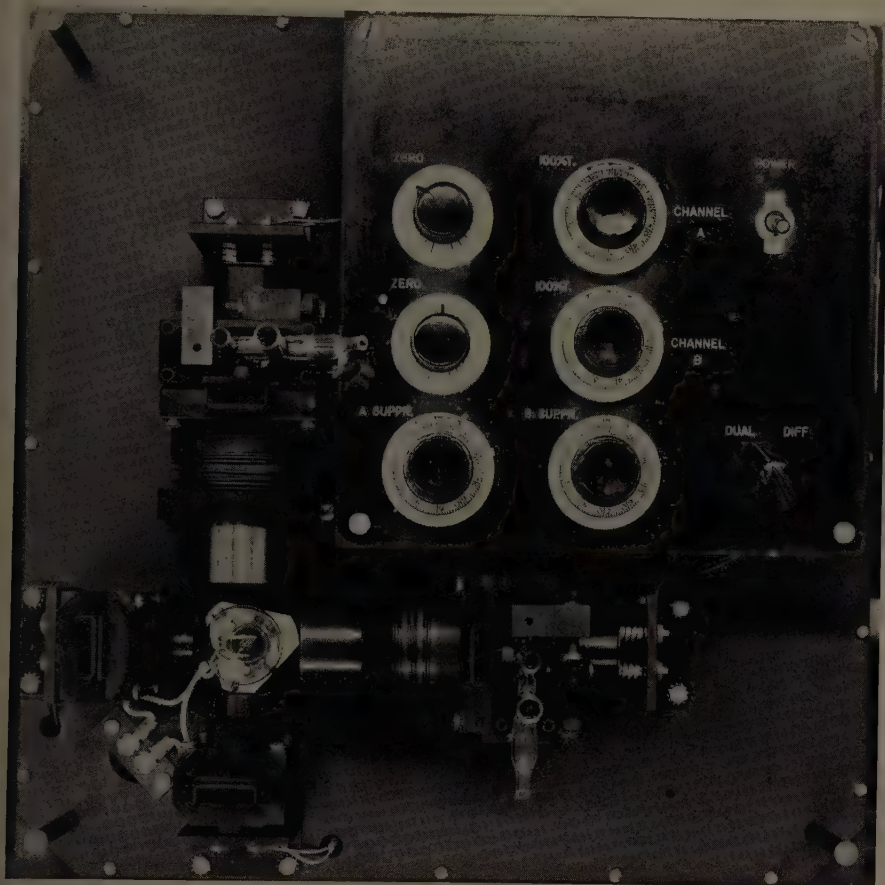


FIGURE 4. Dual colorimeter.

tated the additional step of manually transferring the sample and subtracting one optical density from the other to arrive at an absolute potency. For better automation of fermentation processes, however, the simultaneous determination of sample and blank by a single instrument was desirable.

To accomplish this a dual colorimeter was developed. It is designed to permit either separate but simultaneous recordings of sample and blank or, with the blank as a reference, the recording of only the difference between the sample and blank.

The dual colorimeter (FIGURE 4) is designed for two flow cells, one for the

reacted sample stream and the other for the sample blank stream. The sample blank flow cell is positioned on the reference side of the circuit. Properly phased streams of sample blank and reacted sample flow through the cells simultaneously, and the effect of the blank value is cancelled automatically. The difference between the two streams is recorded in terms of optical density.

The components of the dual colorimeter (FIGURE 5) are designed to occupy a minimum of space. Each of the two flow-cell compartments is provided with its own filter and aperture holder, permitting the construction of symmetrical optical paths for channels A and B. For greater versatility of the colorimeter, each flow cell is provided with a separate reference photocell. Thus, with proper electric switching, the unit can function as two identical colorimeters operating from a common light source. Each of the signal out-

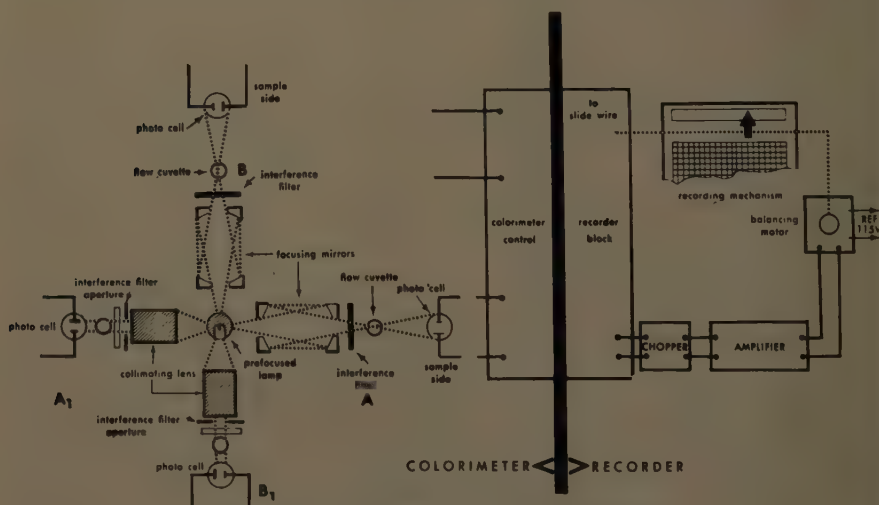


FIGURE 5. Components of the dual colorimeter.

puts of circuits A-A' and B-B' can be connected to either a two-pen or a two-point recorder.

Electrical controls are provided for each circuit, that is, for the differential colorimeter (channels A-B), as well as for the dual colorimeter. In dual colorimetry, channel A controls circuit A-A'; channel B, circuit B-B'. In differential colorimetry, channel A controls the circuitry between A and B, and photocells A' and B' are automatically disconnected from the circuit. A two-position switch permits selection of the mode of operation.

Suppression controls A and B (FIGURE 4) are provided for circuit A-A' and B-B', while for differential operation suppression control A is used for circuit A-B.

Under normal operation the suppression controls are set at 1000, the maximum clockwise position. This provides for zero suppression. Counterclockwise rotation of the controls provides for any degree of suppression of a base line or a given value, and such suppression must be taken into account in interpreting the results.



Minor differences in flow rates between the input system to each flow cuvette can be equalized conveniently by adjusting the length of the tubing, and hence the time en route, between the cuvette and the last time-delay coil. Equalization lengths are determined easily by timing the rates of flow of a dye solution, with an absorption maximum at the desired wave length, through both AutoAnalyzer systems. It is necessary to match exactly the interference filters in both channels of the colorimeter, since small differences in optical densities detract from the precision of the assay.

The differential system employs one colorimeter and a sampler which aspirates sample and blank simultaneously via a sampling crook of double-diameter bore accommodating the two sampling tubes. One pump controls the

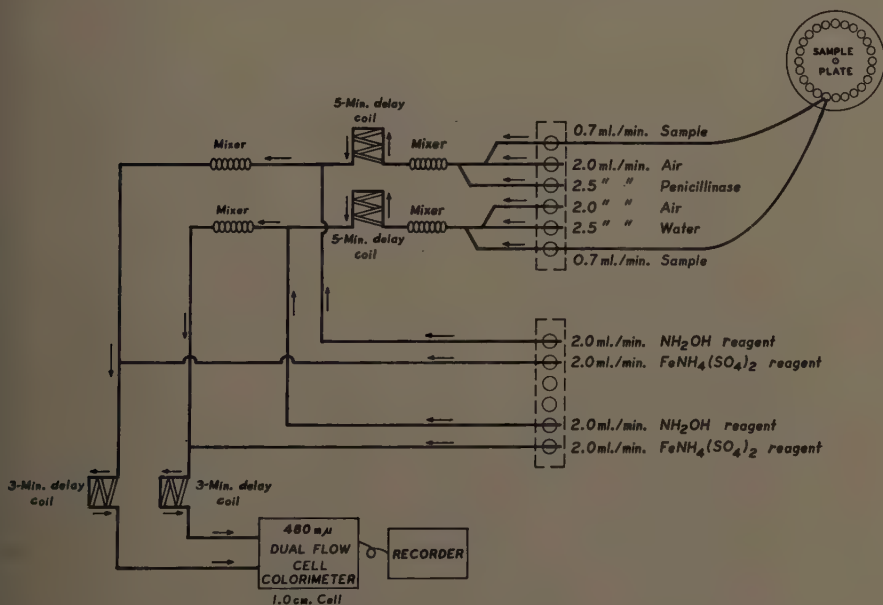


FIGURE 6. Differential colorimeter system.

sampling for blank and unknown, while a second pump controls the reagent flow (FIGURE 6).

To adapt the hydroxylamine method to automatic differential colorimetry, two AutoAnalyzer systems were used which were processed ultimately through the dual colorimeter. The manifolds for the hydroxylamine method were used in the study of the dual and differential systems.

### Results and Discussion

To evaluate the performance of the differential system as compared with the dual system, 2 series of penicillin broth samples were determined on both instrumental systems. Penicillin broths at 2 concentration levels were selected in random order from various fermentation tanks. The data gathered from this comparative experiment (TABLES 1 and 2) show that the instrumental methods agree with each other within an over-all error of  $\pm 3$  per cent. This

error can be attributed to slight differences in the original (dual) system wherein 2 manifolds and separate colorimeters are used. This error is still less than that encountered in the routine determination of penicillin by conventional methods, and is equal to that of the dual system.

TABLE 1  
COMPARISON OF POTENCIES\* AS DETERMINED BY DUAL AND  
DIFFERENTIAL COLORIMETRY

Sample	Potency (dual method)	Potency (differential method)
1	5682	5650
2	4068	4046
3	5726	5514
4	5570	5568
5	5660	5540
6	5782	5812
7	5458	5242
8	5818	5540
9	5858	5704
10	5040	4998
11	5676	5514
12	5908	5866
13	5712	5686
14	5546	5514
15	5644	5622
Average potency	5543	5454

\* Broth samples at high-level potency.

TABLE 2  
COMPARISON OF POTENCIES\* AS DETERMINED BY DUAL AND  
DIFFERENTIAL COLORIMETRY

Sample	Potency (dual method)	Potency (differential method)
1	2625	2608
2	2450	2546
3	2525	2546
4	2525	2639
5	2650	2748
6	2700	2748
7	2525	2561
8	2300	2251
9	2300	2282
10	2200	2158
Average potency	2480	2509

\* Broth samples at low-level potency.

### Summary

The development of a dual colorimeter as an additional component of the AutoAnalyzer instrumental system provides a means for the simultaneous determination of sample and blank. The colorimeter is designed to permit either the separate but simultaneous recordings of sample and blank values or,

with the blank as a reference, the recording of only the difference between sample and blank. The correlation of data obtained by both instrumental approaches is satisfactory and demonstrates the usefulness of the differential method in measuring penicillin potency. In addition, the dual colorimeter can be used to advantage in the continuous monitoring of fermentations for antibiotic production, or wherever the functions of two or more variables can be assessed simultaneously to optimize such processes.

#### *Acknowledgment*

We are indebted to Mary Brilla of the Analytical Research Laboratories of the Squibb Institute for her able assistance in carrying out the experimental work.

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## PARTIAL OR COMPLETE AUTOMATION AS ALTERNATIVES AVAILABLE TO THE ANALYST

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When considering the application of automatic equipment to an analytical method, it is of considerable importance that both the prospective user and the manufacturer collaborate to the greatest extent possible if the most successful conclusion is to be reached. While only the user has fully detailed knowledge of his plant and process conditions, the manufacturer has specialized experience of the capabilities of the apparatus he offers. All this information needs to be combined for the greatest advantage to be gained.

The manufacturer will be anxious to learn as much as possible of the details of the particular application, so that he may propose the most satisfactory combination of his available equipment and techniques. In addition, the user must be prepared to consider the manufacturer's recommendation as to the preferred method of automating certain stages of the analysis when this can be based on the result of experience of other not dissimilar applications.

When discussing an analytical technique with automation in mind, the user must reveal the various detailed steps in the existing method, and their significance to the manufacturer (often under a security agreement to protect the user's interests). It is at this stage that the decision must be made as to whether complete or only partial automation is advisable. In many cases, as illustrated later, it is thoroughly desirable to produce a fully automatic installation; in other cases, consideration of the place for partial automation, such as is given here, is of importance.

The desire to turn routine analyses over to automatic equipment continues to increase, following the demands for speed and reproducibility, in such work. Moreover, it may seem highly desirable to the user to remove from his staff all responsibility for the analytical results. From the manufacturer's point of view this may not be technically impossible; it is one purpose of this paper, however, to illustrate that if this desire is allowed free course it may lead to a degree of automation that is neither economic nor necessary. Close cooperation between the customer and the manufacturer in the manner described will prevent such over-automation from occurring.

To illustrate this, and to show examples of automation of different phases of analytical work, reference will be made to a project in which one company\* was involved. Due acknowledgment is made to this company for its permission to discuss the work to this extent.

During an extended research program associated with the production of penicillin, the firm found it necessary to determine the penicillin in fermentation samples in numbers up to 500 per day. The analytical method chosen for this work, essentially that described by Pan,<sup>1</sup> is illustrated schematically in FIGURE 1. It will be seen from this that the whole procedure is quite lengthy, involving such widely differing techniques as filtration, pipetting,

\* Glaxo Laboratories Ltd., Ulverston, Lancashire, England.



shaking, evaporation to dryness, and absorption measurement. To carry out such work by manual methods would have required several trained operators and, for the quantity of samples mentioned, probably 7 persons would have been needed. The errors that may be introduced into such a system are clearly numerous, yet an over-all accuracy of  $\pm 3$  per cent or better was required.

Here, then, was a case where the use of automatic methods could release a number of workers from tedious repetitive work and also guard against some of the many possible errors. However, because the procedure was so involved, thorough automation would present a formidable though not necessarily an insuperable problem. The agreed approach to this task, therefore, was to automate only those stages following the introduction of the sample, where precision was a major necessity. The remaining intermediate stages, including the transporting of sample tubes from one automatic stage to the next, were to be left to a pair of unskilled assistants.

The stages marked *a* in FIGURE 1 were to be performed automatically; these were: (1) the dispensing of seven different liquid volumes, (2) the transfer of a liquid aliquot, and (3) the final measurement of color intensity by absorption. The installation of the equipment constructed for these tasks is shown in FIGURE 2.

It was convenient to keep the samples in groups of ten for recording purposes. In order to speed the work, three such groups were handled at a time, by mounting thirty tubes at a time in special racks.

Each of 7 dispensing steps was carried out by using an automatic pipette as shown in FIGURE 3, which also shows the 3 sets of 10 sample tubes in individual subracks. The dispensing action is obtained with 3 modified hypodermic syringes, each fitted with solenoid-operated fill and empty valves and adjusted to fill to the required volume. All 3 syringes in a given pipetting unit are connected to the same reagent reservoir and, as the sample tube rack is introduced into the apparatus, a switch is operated by the rack to energize the inlet valves so that the syringes fill under the hydrostatic pressure of the reagent supply (a pressure head of approximately 4 feet of water is required). At the top of its travel each syringe piston closes a pair of electrical contacts in series, so that when all 3 syringes are filled a connection is made to the simple relay control circuit to cause the inlet valves to close and the outlet valves to open. The syringes then deliver the measured volumes of reagent into the first tube in each of the 3 rows of 10. When the delivery is completed, a lamp signal to the operator shows that the rack may be advanced to the second line of 3 tubes where the syringe filling and delivery are repeated.

In the range of syringe volumes available, from 1 to 20 ml. maximum capacity, reagent deliveries are consistent to better than  $\pm 0.005$  ml. of the chosen setting, and all 30 tubes in a given rack can be treated with reagent to this order of accuracy within 90 sec. for 10-ml. deliveries, or within 25 sec. for 0.1-ml. volumes.

With these automatic pipettes, no responsibility for the correct dosage of reagent rests with the operator beyond the simple requirement that he move the rack forward in 10 steps at times indicated by the signal lamp.

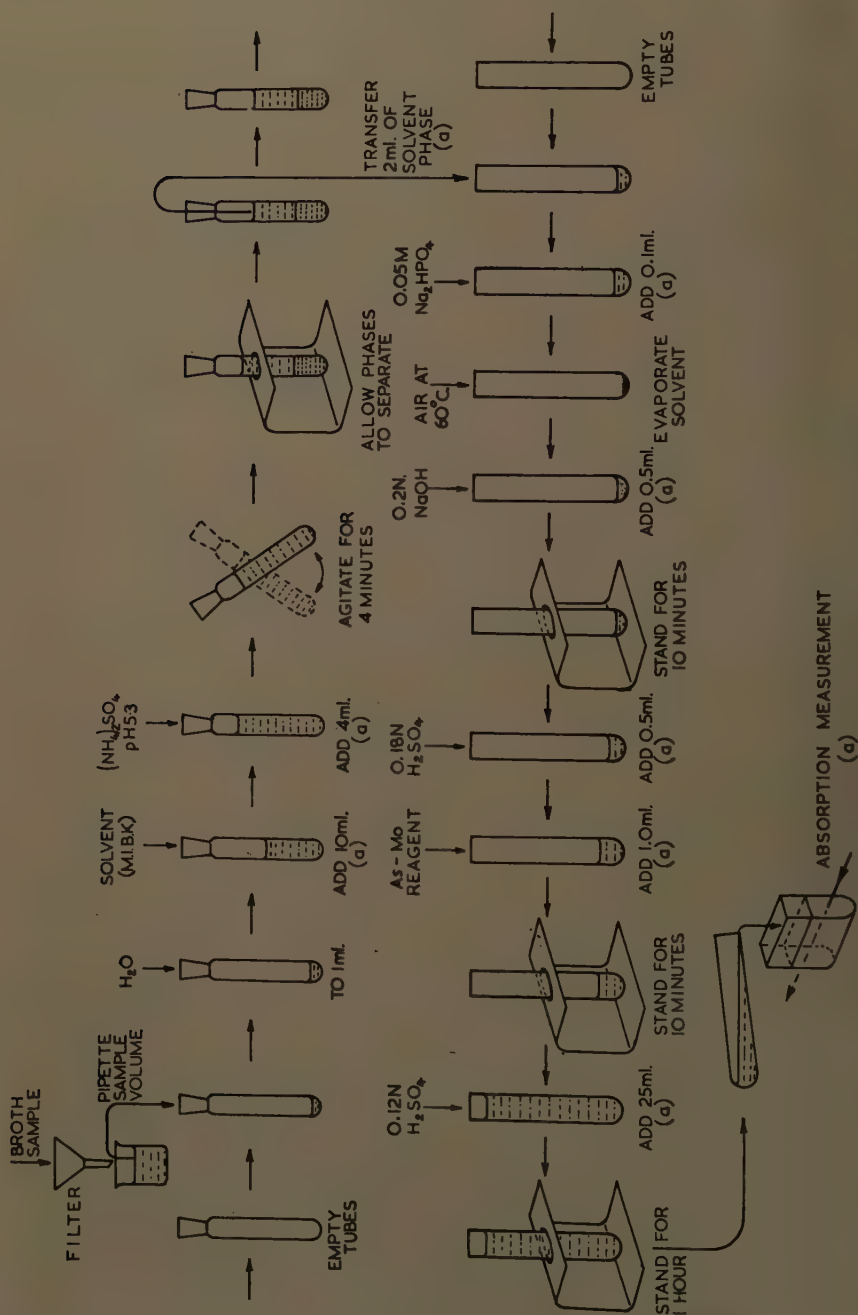


FIGURE 1. Schematic diagram of penicillin assay.



FIGURE 2. Installation at Glaxo Laboratories Ltd.



FIGURE 3. Automatic dispensing pipette.

To obtain the 2-ml. sample from the upper of the two liquid phases that form in the sample tubes after the extraction of the sample into methylisobutyl ketone, a second type of instrument was constructed (FIGURE 4). The left-hand rack contains the sample tubes already treated, and a similar rack next to it contains empty tubes to be filled with extracted samples for the final stages of the analysis. The 2 racks are advanced into the apparatus together, again in 10 movements.



FIGURE 4. Automatic transfer pipette.

When the first indexed position is reached, the racks are mechanically locked in position while the following sequence of events occurs, the various movements being achieved with the use of 3 pneumatic cylinders:

Three syringe pipettes on a frame are lowered together until their jets are at the desired level in the solvent phase in the sample tubes, and their pistons are then raised to take up the 2-ml. volumes required. Next, they are raised together, transferred to the right until the jets are above the corresponding 3 empty tubes, and lowered, at which stage the syringes discharge their contents. Finally, the empty syringes are raised and returned to their rest position above the first rack. When a lamp has signalled the completion of the movements, the rack may be advanced by the operator into the next indexed position, and the cycle of movements repeated.

By this means the 2-ml. aliquots from 30 sample tubes are transferred into the receiving tubes in approximately 2 min. with an accuracy of  $\pm 0.5$  per



cent, a speed and precision beyond the capability of an operator to maintain over long periods using conventional pipetting methods.

To determine the absorbance of the resulting final colored solutions it was found particularly necessary to adapt the special recording absorptiometer



FIGURE 5. Absorptiometer guide for sample tubes.

seen at the right in FIGURE 2, for work with samples presented in test tubes in groups of 10 at a time. The only demand made upon the operator at this stage was that he take each tube in turn from a rack and insert and raise it into a guide in the front panel seen in FIGURE 5. Again, a lamp signal was re-

quired to indicate when the instrument had completed and recorded the measurement of absorption, leaving the operator to withdraw the tube and replace it by the next, at a rate possibly as high as 250 tubes per hour.

The absorptiometer of this apparatus, situated immediately behind the top of the panel shown in FIGURE 5, is illustrated in FIGURE 6. This shows the top of the intake guide at the lower left, together with the glass jet that projects down into the liquid in the tube as it is raised. This jet connects to the inlet at the base of one glass-windowed cuvette, and a simple bellows pump is con-



FIGURE 6. Photoelectric absorptiometer (covers removed).

nected to the top of the cuvette. The other components of the absorptiometer, comprising the lamp, the vacuum-type photocells (only the reference photocell at the extreme right is exposed), and the reference cuvette also may be seen.

When a tube has been inserted into the guide, it is gripped firmly by the action of a solenoid, seen at the bottom of FIGURE 6, so that the tube cannot be withdrawn until the measurement has been completed. At the same time a synchronous motor is started, and this operates switches set to control the following sequence:

First, the bellows pump is extended to cause sample liquid to be drawn from the tube to fill the cuvette. The photocells are then connected via a

simple amplifier to the recorder circuit, and the ratio of the two photocell signals is recorded as the percentage absorption of the colored solution with respect to the absorption produced in the reference cuvette. Successive results are printed on a standard 10-in. chart alongside corresponding identi-

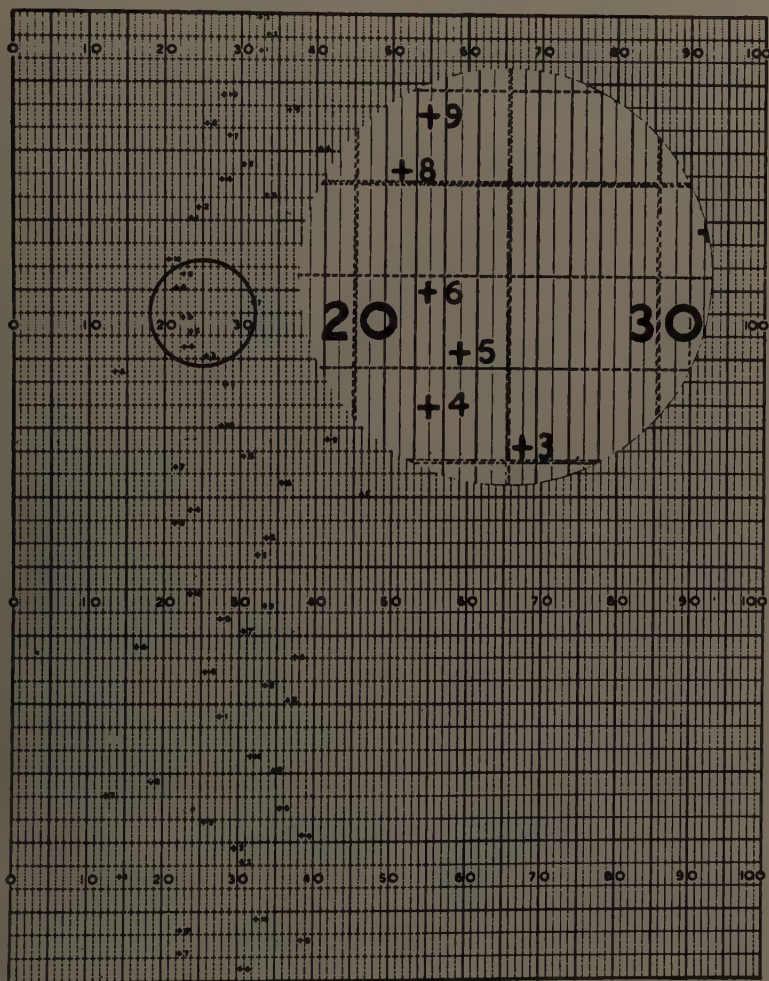


FIGURE 7. Recording absorptiometer chart record.

cation numbers from 1 to 10, as shown in FIGURE 7. Between each group of 10 results, a section of the chart corresponding to the unused recorder positions 11 and 12 is left blank to separate the results into their respective groups for ease of identification. Measurement accuracy at this stage is  $\pm 1.0$  per cent.

After sufficient time has elapsed for printing the result, the bellows action is reversed to expel the colored sample back into the sample tube, and the

lamp signifies completion of the measurement. Finally, as the operator withdraws the tube from the guide, the tube depresses a lever switch at the lower end of the guide (FIGURE 5). This starts a pump that forces a momentary air purge through the now empty cuvette and intake jet, in order to remove the few drainage droplets that might still remain. A contamination test has shown this method to be more effective and less troublesome than efforts to rinse the cuvette between samples with water or other liquid.

The use of automation in this way for this application is described by Green and Monk,<sup>2</sup> who give full details of the method and report that the over-all precision of the procedure as automated was found to be  $\pm 2.5$  per cent, an improvement of  $\pm 0.5$  per cent on their original requirement.

It is significant to consider here the value of such an installation, which requires only two relatively unskilled operators and can result in the achievement of the desired accuracy. If one contrasts the complexity of instrumentation used (remembering that each unit is a separate and therefore easily serviced item) with what would have been involved if the whole technique of FIGURE 1 had been automated, the advantage in using just the optimum amount of automation can be seen. It is not possible to estimate accurately what would have been the relative cost of partial as opposed to complete automation of the method, since the latter was not attempted, but it is clear that complete automation would have been a prohibitively expensive project.

Nevertheless, as already mentioned, there are numerous other applications where it is not only desirable and economical, but even essential, that the whole analysis be rendered completely automatic. This is almost invariably the case where continuous streams of plant liquors are to be analyzed. Two examples will be sufficient to point out the contrast with the partial automation already described.

The first of these, again employing liquid/liquid solvent extraction, but on a continuous basis, is shown in FIGURE 8. This illustrates a multiple input instrument for monitoring uranium concentration in five aqueous streams at one of the United Kingdom Atomic Energy Authority's plants. Here again, color densities dependent on the concentration in each stream are monitored in turn by the two absorptiometer-recorder units at the right-hand side of FIGURE 8. These units are used interchangeably to permit routine maintenance without shutting down the instrument, and also to permit continuous monitoring of one stream alone while corrective plant action is carried out, should the concentration be found to be excessive in that stream.

The glass components of the extraction unit are shown in FIGURE 9, with two vertical extraction columns below each of the stirrer motors. The sample, solvent, and reagent flows are admitted to the input limbs of the columns from the four controlled-rate drip-feed injectors on the smaller panel at the top of the illustration.

In the first (right-hand) column, the uranium is extracted from the aqueous stream into tributyl phosphate; in the second column it is backwashed from the solvent by ethylenediaminetetraacetic acid (EDTA) and then mixed with ammonium thioglycollate to develop the color to be examined absorptiometrically. Continuous extraction is achieved in these columns, since in each case



the lighter solvent phase is introduced at the bottom limb of the column and thus passes up through the heavier medium. A 6-mm. glass rod located along the axis of each column is fitted in polyethylene bearings at the top and bottom of the columns. The rods, rotated by the stirrer motors at 2500 to 3000 rpm, promote thorough intermixing so that highly efficient extraction takes place.

The outflows from each extraction panel are collected in individual vessels that are emptied automatically in turn, at 3-min. intervals, to pass their con-

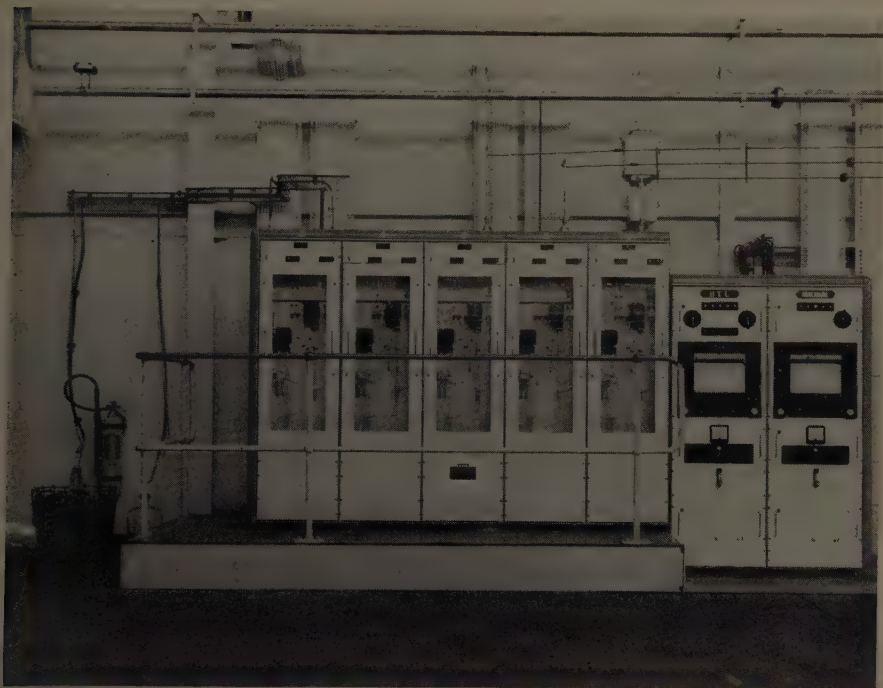


FIGURE 8. Five-input uranium concentration recorder.

tents to the absorption measurement cell. Thus a record is presented for all 5 sample streams every 15 min. on a 24-hour basis.

Another major technique used by the analyst is that of titration; in many applications this must be fully automatic. FIGURE 10 shows the two units that comprise one such installation. Designs of this type are adaptable to the whole range of electrometric and also colorimetric end points.

The equipment is separated into two parts so that the electrical control and recording components may be kept clear of the chemical section. The latter, in the example illustrated, was equipped with an exhaust ventilation system to remove toxic fumes which could arise under certain conditions. The actual titration panel is seen in FIGURE 11. The reaction vessel is at the bottom, the sample- and reagent-dispensing pipettes to the left, the burette and

meniscus-finding device in the center, and the normality compensation mechanism to the right.

The full automatic sequence of such titration equipment for plant installations frequently involves as many as twenty-five individual steps. These



FIGURE 9. Continuous liquid/liquid solvent extraction unit.

include sample-line flushing to procure a contemporary sample, the taking of sample and any necessary reagent volumes, stirring, titration to a predetermined end point, automatic recording of the result, and all final draining, washing, and refilling operations.

The titration result may be recorded directly as concentration of the sample constituent under study, rather than as merely the milliliters of titrant necessary to reach the end point. In this way the results, presented either digitally or on a standard strip or circular chart recorder, may be examined later without further calculation, and direct control action from the recorder movement may be used to correct deviations from the desired plant operating conditions.

The two examples just described are typical of the manner in which an entire analytical procedure can be rendered automatic. Such installations are



FIGURE 10. Automatic recording titrator.

capable of functioning unattended as long as sample is available and provided that reagent stocks are replenished as necessary and a number of simple maintenance checks are made at appointed intervals.

The final assembly for the automation of a particular project necessarily must consist of several individual automatic devices that will perform the various stages of the procedure. Although these are often built to form a single self-contained automatic apparatus, it may be possible to group them more favorably into separate units and, as in the penicillin project quoted, to use a certain proportion of human labor to gain the greatest advantage.

The analyst thus has the choice, when automation is desired, of applying it either fully or partially. The realization of the existence of these individual



FIGURE 11. Burette panel of automatic titrator.



devices or "bricks" for automation will enable him to come to an agreement with the manufacturer as to how they may be most profitably combined.

### *Summary*

The part played by automatic instruments in the work of a process or plant analyst continues to increase, and it is now possible for whole analytical techniques to be performed by instruments working independently of human attention. However, it is necessary to examine the requirements of each application to decide what degree of automation should be applied to a given analytical method. Indeed, it is possible to over-automate a procedure to such an extent as to render the over-all scheme uneconomical.

This possibility is illustrated by detailed reference to a requirement arising from a large research program associated with the manufacture of penicillin. The usefulness of automating only certain vital stages of such a program is shown in contrast to what might have been expended had the project been rendered completely automatic.

Alternatively, instances are quoted where automation of all stages of an analysis not only was desirable from considerations of the over-all accuracy demanded, but was also fully justified economically because of the continuous nature of the applications.

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## RECENT DEVELOPMENTS IN AUTOMATIC COLORIMETRIC CHEMICAL ANALYSIS INSTRUMENTS

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Continuous on-stream instrumentation has been receiving increasing attention and study during the last five to ten years. Previously, use of this form of instrumentation had been limited almost to measurement of physical variables or electrical characteristics of the stream. The wide interest in infrared analyzers and chromatography is indicative of this.

The control laboratory, however, has remained a part of the control loop for many variables because, until recently, all too many of the analyses of these variables did not lend themselves to measurement by any type of industrial instrumentation available. A need existed, therefore, for an instrument or a group of instruments designed as rugged industrial devices that would eliminate or minimize routine, repetitive laboratory chemical analysis. Such instrumentation would make these analyses with the greater frequency required for continuous control and would be adaptable to feedback and automatic correction loops within the system.

This paper is concerned specifically with one class of on-stream, colorimetric industrial analyzers developed for measurements in which the color is the result of a chemical reaction. Development of such instruments started 6 years ago in the German Federal Republic, where it progressed concurrently with its development in the United States during the past 3 years. This form of instrumentation is now in use in many plants in Europe. Over 500 instruments have been sold and installed during the past 3 years. In the United States, a corresponding instrument, the Quantichem† has been developed to a still higher degree of refinement before commercial marketing. It has been field-tested also, and a representative number of field installations indicate that it has reached the point of practical industrial use.

The commercial instrument has little resemblance to the original laboratory assembly of glassware. The original design objectives were:

- (1) A mechanical system capable of metering with a high degree of repetitive accuracy exact volumes of both sample and reagent or reagents. The system was to be constructed of rugged materials selected to withstand corrosive effects of chemical reagents required. The instrument was designed for industrial use, not for the laboratory.
- (2) An optical system that, with the proper filters and photocells, would produce results of highest accuracy and reproducibility consistent with the accuracy.
- (3) An electrical system for optimum amplification of photocell signals for recording, and for feedback controls.

\* Now with Milton Roy Co., St. Petersburg, Fla.

† Milton Roy Co., Philadelphia, Pa.

(4) A chemical method of analysis that, in its adaptation, would permit the preparation and storage of chemicals without deterioration for periods varying from one week to one month. The method of analysis used must combine the mechanical, optical, and electrical systems in a completely reliable industrial instrument that would require a minimum of maintenance.

The instrument could have been designed either for continuous or semi-continuous analysis, or for repetitive cyclic analysis. Since continuous analysis offered little advantage, if any, from the standpoint of time elapsing between sample procurement and read-out, it was decided to proceed with the cyclic design because of the higher accuracy possible and the flexibility as to sample spacing, which would permit more frequent sampling close to the time

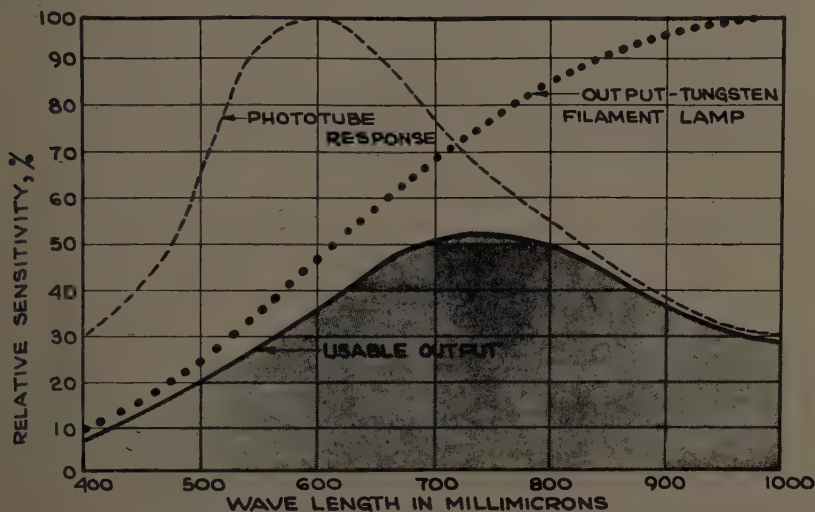


FIGURE 1. Curves of typical responses for light source and phototube.

of an anticipated process change. Furthermore, cyclic analyses obviously could be more economical of costly chemical reagents required for the tests.

FIGURE 1 illustrates a typical set of response curves for a light source (*dotted line*) and a photocell (*dashed line*). From this, it is possible to determine the useful range of the instrument (*solid line*). In this case, the normally useful range of the combination would lie between 400 and 1000 mμ. Similar curves would be drawn for the other components of the system.

In order to make use of the instrument for chemical analysis, it is necessary to be able to measure the percentage transmittance at a specific wave length. An optical filter is used for this purpose. In essence, a filter is a selective shade that ideally completely blocks out light of all wave lengths except the desired one. In practice, a curve similar to FIGURE 2 results. The minor deviations due to transmittance at wave lengths other than the desired one are usually unimportant.

Why is colorimetry preferable to other methods of analysis? Recent work, particularly in the power and nuclear fields, has required accurate determina-

tion of materials in solution at concentrations of less than one part per million and, in some cases, considerably lower. At these low concentrations almost all materials follow Beer's law. In many cases, colorimetric analyses can be made accurately at one-thousandth or less the concentration that can be detected by conductivity or *pH* value. Colorimetric analysis usually can resolve ion species in the presence of other material at several thousand times the concentration of the ion being analyzed.

### *Instrument Development*

The commercial development of a colorimetric on-stream chemical analyzer was initiated in about 1952 by Hans Fuhrmann at the firm of Bran & L  bbe\*

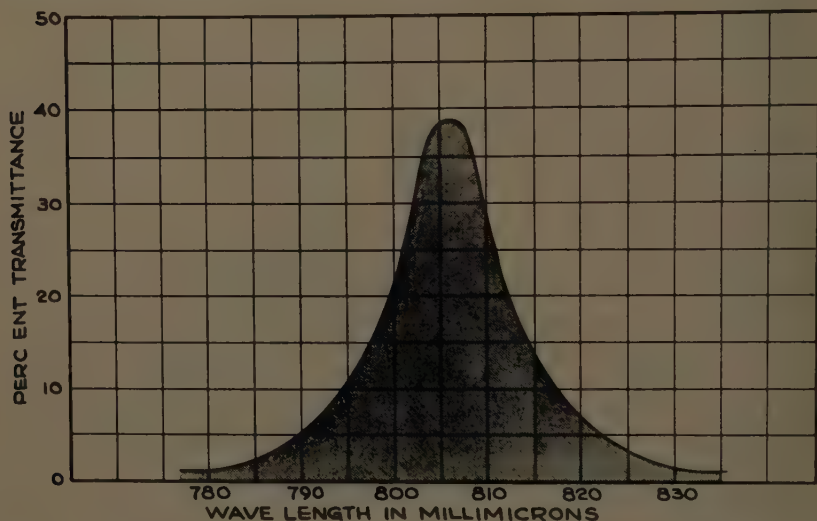


FIGURE 2. Effect of narrow-band interference filter in producing monochromatic light.

in the German Federal Republic. The first instruments<sup>1-3</sup> were designed specifically for the determination of residual hardness in water following a zeolite or an ion-exchange process. Many commercial installations of this instrument were made in power plants to monitor water softeners, to signal any breakthrough of hardness, and to initiate the regeneration process.

FIGURE 3 illustrates typical transmittance curves covering the water-hardness ranges of 0 to 3.5 ppm and 0 to 7 ppm calcium carbonate. The two curves were obtained with two different monochromatic light filters. It is obvious that the read-out scale of the instrument must be designed to correspond to curve for the light filter selected. Fixed quantities of sample, indicator, buffer, and magnesium dipotassium ethylenediaminetetraacetate ( $\text{MgK}_2\text{EDTA}$ ) are employed. The color developed, varying from blue to violet to red-violet to red over a range of 0 to 7 ppm total hardness, is a

\* Bran & L  bbe Wasseraufbereitung Maschinenzund Apparatebau, Hamburg 39, German Federal Republic.



measure of the quantity of calcium and magnesium in the sample. Eriochrome Black T, with a sufficient amount of the complexing agent  $\text{MgK}_2\text{EDTA}$ , is used as the indicator. The sole function of the  $\text{MgK}_2\text{EDTA}$  is to chelate the free calcium ions in solution and release an equivalent amount of magnesium ions, the Eriochrome Black T being sensitive only to the magnesium.

Following an initial and thorough study of the first German instruments, development of similar colorimetric analyzers was undertaken in the United States by the Milton Roy Co. Design objectives were established as already outlined. It was felt that the ultimate instrument should eliminate, as far as possible, internal laboratory-type glassware and should provide positive, accurate volumetric measurement of both sample and reagents. The present design of the Quantichem has achieved these objectives.<sup>4</sup>

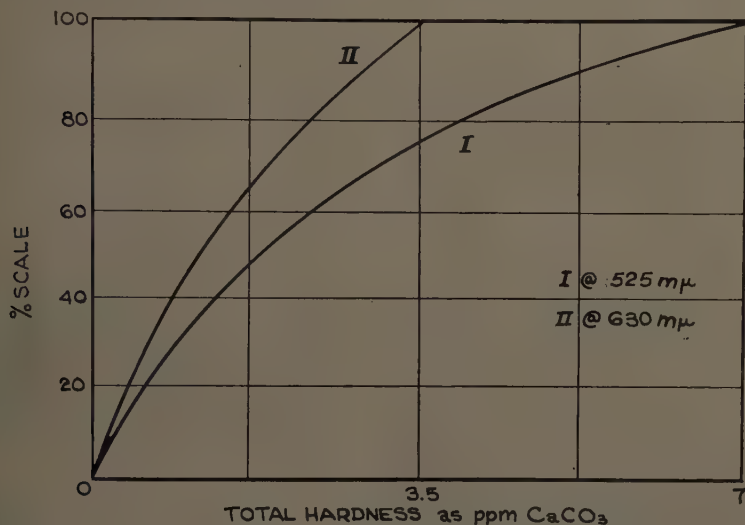


FIGURE 3. Effect of wave length on light transmission for hardness measurement.

The two units that comprise the instrument (FIGURE 4) are the analyzer and the recorder. The analyzer houses the sample- and reagent-metering pumps, the sample cells, the light source, light filters, photocells, and the program-timing system. To facilitate maintenance by the operator, three tell-tale lights are located prominently on the face of the analyzer unit below a master program timer. The center lamp lights when the instrument is on power. The light on the right (FIGURE 4) flashes when the cells need cleaning. The light on the left flashes if the light source in the analyzer fails.

The master program timer, whose dial is visible on the face of the instrument, controls all steps of the analysis. A manual-automatic switch placed immediately below the timer enables the operator to stop the analysis at any point in the circuit and check the functioning of the instrument by manually operating the timer.

The recorder is a standard instrument, modified to meet the requirements of this application. It contains the amplifier, bridge circuits, and the DC-AC

converter. AC amplification is used because of its inherent stability and freedom from drift. This feature permits a single calibration of the instrument, eliminating periodic standardization required by DC amplification systems.

The schematic of the Quantichem in its simplest form, as designed for water-hardness determination, is shown in FIGURE 5.

A unique pneumatic timing system that works on the principle of the player piano is employed. The air supply at 20 psi is admitted to a manifold and flows through small orifices to the individual control lines. Each line terminates in an O-ring Teflon nozzle, sealed by the solid surface of the program-



FIGURE 4. Quantichem colorimetric end-point analyzer and recorder.

ming drum, and the other end of the line terminates at either a pressure-actuated electrical switch, a pressure-actuated diaphragm valve, or a pressure-actuated reagent pump. The programming drum (FIGURE 5) makes one revolution for each cycle of analysis. At the appropriate time in the cycle, when a valve is to be opened or a pressure-actuated switch energized, air is released from the individual control line through an orifice or a slot in the programming drum so that the pressure drops in that line, the air pressure build-up at the small orifice being insufficient to compensate for the loss of pressure through the programming drum. This system of programming has simplified valve design and functioning of the instrument. Furthermore, electrical controls are minimized in what might prove to be somewhat corrosive atmospheres, facilitating maintenance.

At the start of the cycle, clean sample, filtered if necessary, flows via the pressure-regulating valve through both the sample cell and comparison cell, filling the cells to overflowing with known volumes of sample. The initial sample flow acts to flush the previous sample from the cell and to replace it with sample representative of the process stream. The sample inlet valves then are closed by the program timer.

The zeroing relay and zeroing motor now are energized to standardize the bridge circuit automatically. The purpose of standardizing this portion of the system in each cycle is to compensate for irregularities due to such factors as sample turbidity, coating of cell walls, and dust on lenses. Although these problems have not proved severe in practice, standardization nevertheless is

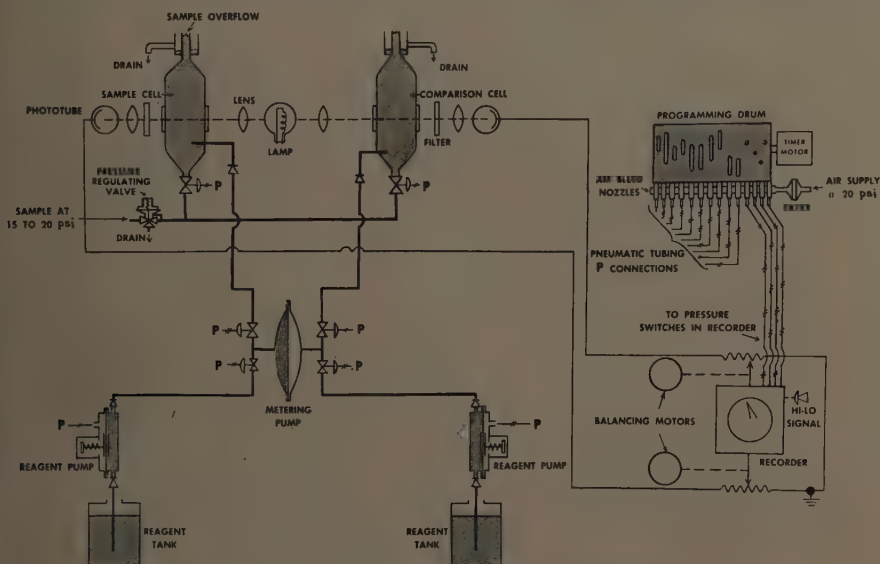


FIGURE 5. Schematic design of hardness analyzer.

incorporated in every cycle. Tests have shown little appreciable drift or change from standard zero on two-day tests. The standardization motor is connected to an adjustable bridge resistor. Since the sample cells are filled with clear water, both phototubes should be subjected to the same amount of light. If there is any variation due to dirt, turbidity, aging, or other factors, there will be a slight unbalance signal, and the amplifier will drive the standardizing motor in the proper direction to balance the bridge circuit. If the bridge cannot be balanced by moving the slide wire to its extreme position, a little cam operates a contact that lights the "clean-the-cells" alarm. The use of such a null-balance type of circuit provides maximum sensitivity in the system.

The design of the metering pump used for measuring the reagents is of interest. This pump consists of two identical segmented spheroidal cavities clamped together, with a slack diaphragm between them. In operation, reagent from the first reagent tank, under pressure from the air-actuated reagent

pump, forces reagent through the first inlet valve behind the slack diaphragm. This forces the diaphragm to the opposite side of the cavity and completely displaces the previously measured reagent from the second reagent tank to the point of application in the comparison cell. The reagent metered to the sample cell is Eriochrome Black T plus EDTA, used to give a 0 hardness reference color. By action of the other inlet and outlet valves on the metering pump, the second reagent is fed behind the diaphragm, forcing the metered quantity of the first reagent to the sample cell. The cavity is designed to meter exactly 2 ml. of reagent with one movement of the slack diaphragm within the pump cavity. This metering is exact and will deliver the desired quantity of reagent to within a fraction of 1 per cent. It has been found that with a single reagent, tangential injection into the sample cell without the use of a mixing motor assures adequate mixing and reaction.

In the programming a sufficient time interval is allowed for proper color development. During this interval, light of different intensities falls on the zero-cell and measuring-cell phototubes and, the bridge is unbalanced. As in the zeroing cycle, a second balancing motor, that drives the chart pen and a second balancing potentiometer in the bridge circuit, drives in the proper direction to rebalance the bridge. The recorder then indicates and records the results of the analysis. Limit switches with alarm contacts adjustable to any desired concentration are provided; these can actuate high- or low-concentration alarms such as horns, bells, or lights. Following recording, the instrument is ready to repeat the cycle, beginning with flushing of the sample cells. A cycle for water-hardness testing can be completed as often as every 3 min. or it can be extended, depending on the requirements of the individual plant. A 6-min. cycle is normal; with this timing, 480 ml. of each reagent is required for each 24 hours of continuous operation. The standardized reagents are supplied in 5-gal. polyethylene-lined containers so that each set of containers holds reagent sufficient for over a month's operation at this cycling rate.

#### *Determination of Dissolved Oxygen*

The determination of oxygen dissolved in boiler feed water is most important and can be achieved with this type of instrument. FIGURE 6 shows the adaptation of the Quantichem to the indigo carmine method of analysis for 0 to 30 parts per billion (ppb) dissolved oxygen. In this case, the sample flows through a closed cell to avoid any contact with atmospheric oxygen and with sample and comparison cells containing fixed volumes. This is accomplished by building into the top of each cell a closure consisting of a diaphragm held in place by air pressure exerted by a piston. Following the flushing cycle and the entry of the desired amounts of sample into the two cells, the pressure is released by the piston, the diaphragm is flattened, and the cell volume expands to accommodate the introduction of reagent. In this case the reagent is indigo carmine and alkali. The indigo carmine and alkali must react in the accumulator and reaction cells before being introduced to the sample, where a specific, quantitative color reaction to the dissolved oxygen develops.



*Analysis for Silica*

In silica analysis,<sup>5,6</sup> one of the more complex applications of the instrument, four specific reagents are metered to the zero and sample cells (FIGURE 7). The first reagent is a buffer to maintain the  $pH$  as close as possible to 1.7. An-

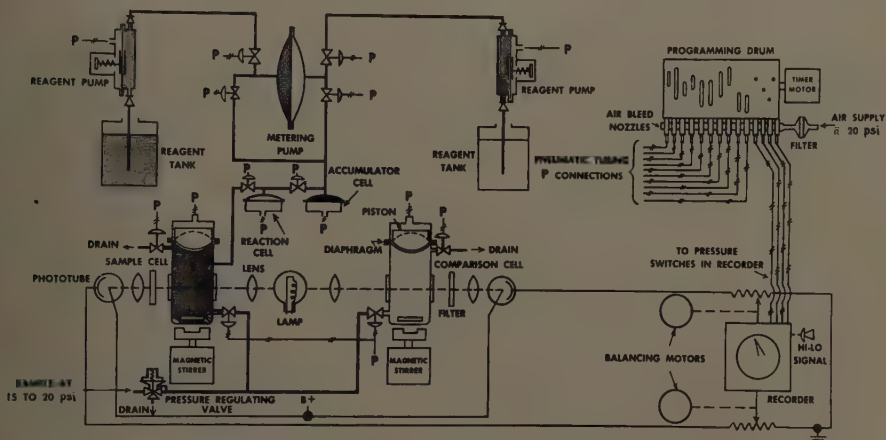


FIGURE 6. Schematic design of oxygen analyzer.

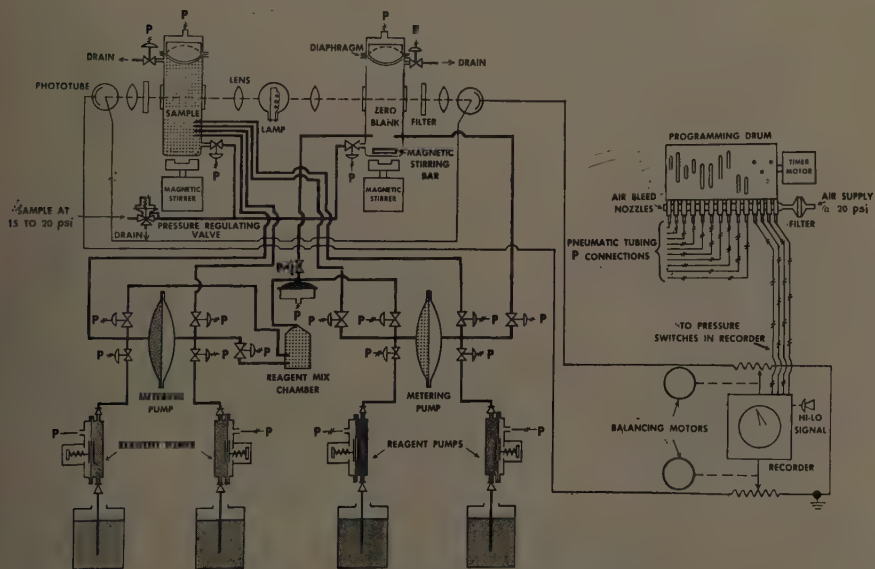


FIGURE 7. Schematic design of silica analyzer.

other reagent is ammonium molybdate. At low  $pH$  the molybdate ions polymerize to form the complex ion,  $(Mo_6O_{21})_6$ , which then forms heteropoly-molybdic acids with the silica and phosphorus in solution.

The third reagent, a complexing agent, then is metered to both cells and

attacks the phosphomolybdic acid, destroying it completely. The reagent has no effect on the silicomolybdic acid. Finally, a reducing agent is added, resulting in the formation of colloidal molybdenum blue. This reaction always goes to completion, and therefore the color intensity is a direct measure of the soluble silica present.

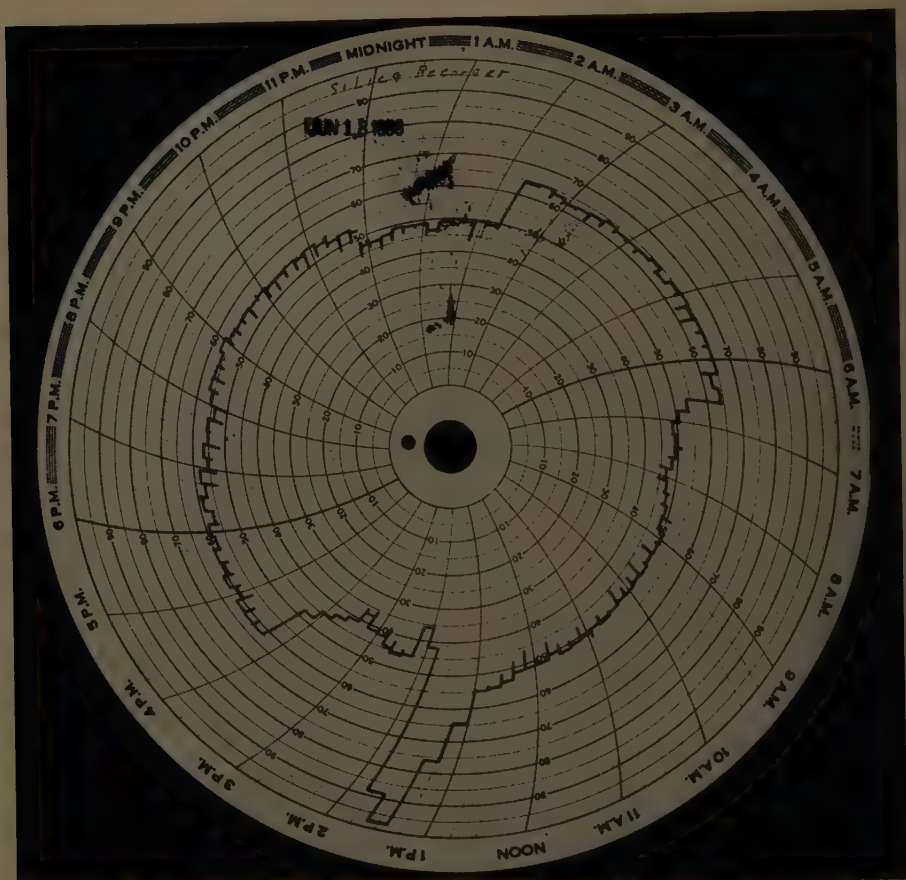


FIGURE 8. SiO<sub>2</sub> chart for Philadelphia Electric Company.

For the blank cell comparison, chemical reagents are fed to a cell to complete their reactions before being fed to the zero cell. Therefore they introduce to that cell only intrinsic color or color due to any trace of silica included. The color difference between the sample and the comparison then will be due only to the silica content of the sample. Each complete cycle normally lasts 12 min. The range of the instrument is normally 0 to 50 ppb, and a change of 1 ppb in the water may be detected accurately.

As the result of refinements in design, the Quantichem is capable of approximately five times the sensitivity of its German prototype. It has been under test and refinement for more than a year and is now in commercial production.

Quantichems have been installed for the determination of hardness and silica in boiler feed waters. FIGURE 8 is a typical  $\text{SiO}_2$  chart from an analyzer installed in a large public utility power station. The variation of silica from the ion exchanger with the rate of flow through the exchanger is evident at, for

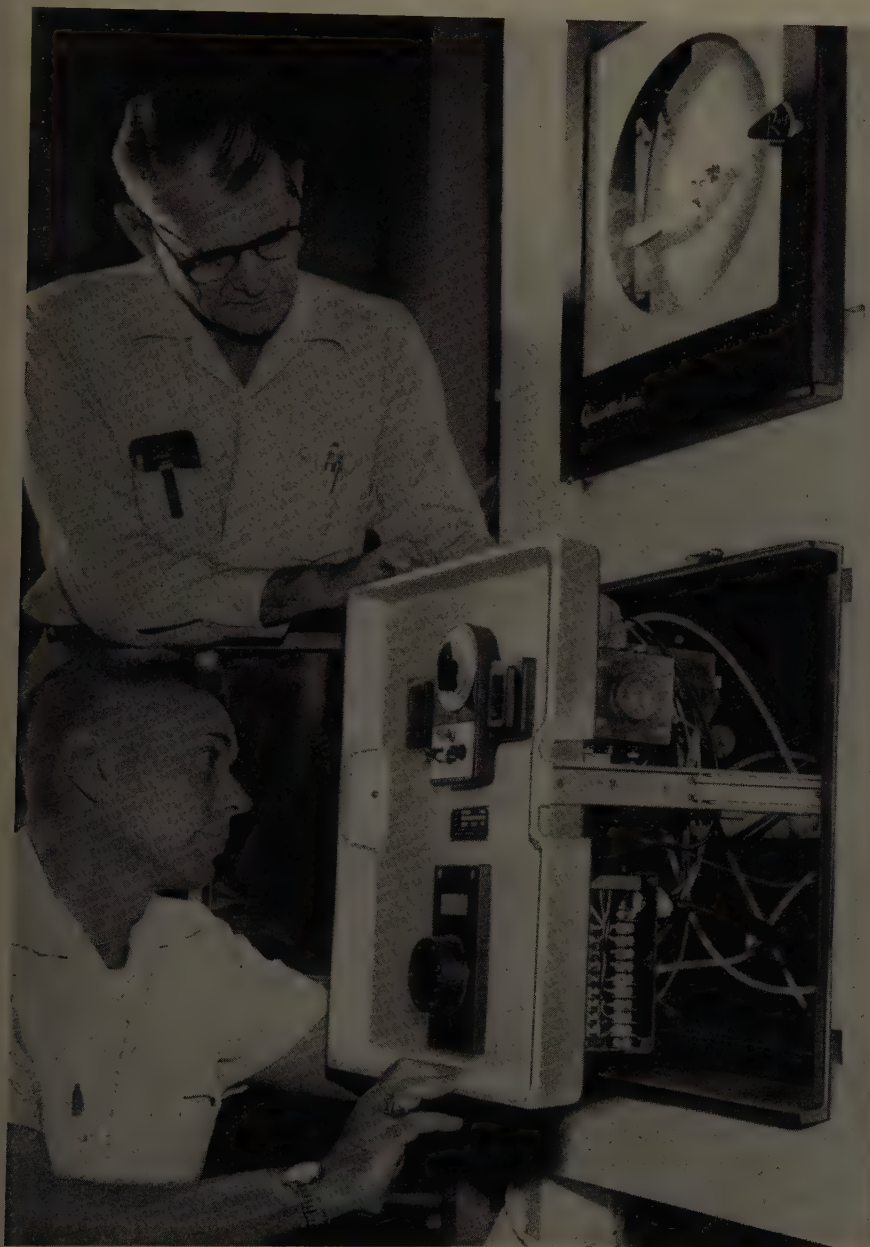


FIGURE 9. Dissolved oxygen analyzer at Florida Power Corporation.

example, 2:20 A.M. and 6:35 A.M. on the chart. The instrument recorded the first breakthrough of silica at 1:00 P.M., signalling the operator to remove that unit from the line for regeneration.

Dual-range silica units can be furnished. For example, an instrument with the ranges of 0 to 50 ppb and 0 to 500 ppb can be designed, simply by modifying the resistances within the instrument. Other silica units can be furnished with a dual range of 0 to 50 and 0 to 2500 ppb. In the latter case, the more silica-laden water (boiler water) is diluted accurately by a proportioning system with controlled volume pumps to 50:1 with silica-free water obtained by passing condensate through a mixed-bed cartridge at the instrument.



FIGURE 10. Picture of Belgian Chem-O-Matic Analyzer.

The instrument thus "sees" water in the same  $\text{SiO}_2$ -range as the water from the large ion exchangers.

FIGURE 9 shows an installation at a large public utility plant in Florida for determination of oxygen dissolved in boiler-feed and condenser water.

### *Applications*

One instrument can be used for a large number of different analyses by appropriate changes in program timers and monochromatic light filters, provided the original instrument is supplied with sufficient number of metering reagent pumps. A maximum of four reagents is required, and all interfering ions in the system being analyzed can be eliminated within a four-reagent system or by an ion exchange.

Procedures are available for the use of the Quantichem in the following colorimetric determinations without pretreatment of sample and with appro-



priate instrument chemistry: Free chlorine, chlorides, chromium, copper, cyanide, fluoride, formaldehyde, hydrazine, iron, dissolved oxygen, orthophosphates, sulfites, dissolved silica, and total hardness ( $\text{Ca} + \text{Mg}$ ).

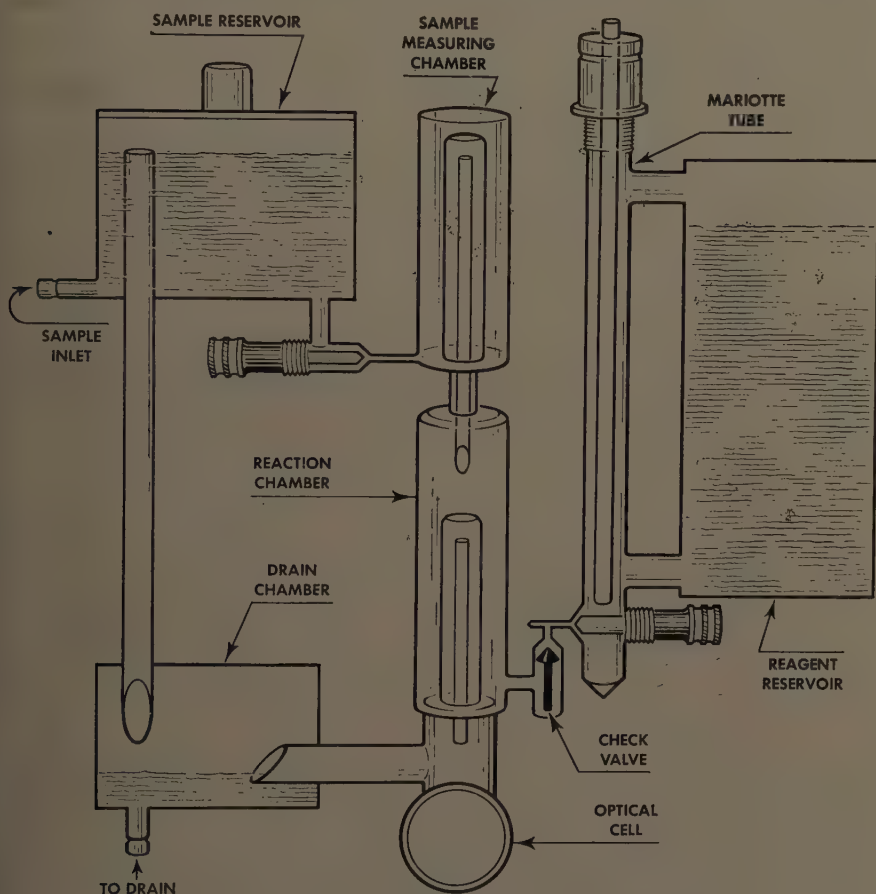


FIGURE 11. Schematic of Belgian Chem-O-Matic Analyzer.

### *"Go-no-go" Colorimetric Analyzers*

The instruments described above are the most refined of the on-stream colorimetric chemical analyzers available at present. However, there exists also a need for simple, inexpensive instruments of a "go-no-go" design. Typical of these is a simple instrument of Belgian design, the Chem-O-Matic\* (FIGURE 10), that works entirely on hydraulic principles. Sample and reagent flow is entirely by gravity.

The sample-measuring chamber (FIGURE 11) is filled by regulated flow of sample to the point of discharge. It is then siphoned into a reaction chamber,

\* Interindustrie, Brussels, Belgium. Available through Milton Roy Co., St. Petersburg, Fla.

where it is mixed with a previously measured volume of colorimetric indicator. The mixture from this chamber in turn overflows via a siphon into a visual optical cell. Sample is measured as the contents of the measuring chamber, approximately 18 cc. Reagent is allowed to flow from the reagent reservoir to the reaction chamber through a small check valve until it reaches a level corresponding to the bottom of the mariotte tube. The effect of this tube is to bring air pressure to a constant level regardless of variations in the reagent reservoir pressure. The resulting color can be seen and compared with a color chart or with color standards. The instrument can respond also to simple photoelectric signals for a "go-no-go" alarm signal or chemical feed system. Such instruments can be employed for colorimetric  $pH$ , hardness in water, phosphate, and other simple colorimetric analyses.

### *Conclusion*

The instruments described are new tools for on-stream chemical analysis and control based on variables hitherto controllable only by laboratory procedures. Many more applications of chemical end-point colorimetric analysis are possible than have been described. The development and field-testing of the present instruments now are so advanced that their ultimate success and commercial acceptance can be promised.

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## AN AUTOMATED METHOD FOR DETERMINATION OF TERRAMYCIN

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Terramycin (oxytetracycline) reacts with ferric ion in acid solution to produce a colored complex with an absorption maximum at approximately 420  $m\mu$ . The reaction obeys the Beer-Lambert law over a wide range of concentration and has been used as the basis of a colorimetric method of assay by Monastero *et al.*<sup>1</sup> Over the years, the antibiotic has been found useful in many infectious conditions in all types of patients; hence it appears in a wide variety of pharmaceutical formulations and mixtures. Adequate quality control of these preparations requires a large volume of samples for chemical analysis. An automated method for the routine colorimetric determination of Terramycin obviously would save time and expense and release trained personnel for other laboratory duties.

The AutoAnalyzer,<sup>\*2,3</sup> a recent development in instrumentation for the automation of colorimetric analyses, has been used in the determination of streptomycin and penicillin.<sup>4</sup> This instrument system is controlled by a multichannel, peristaltic proportioning pump that accurately delivers fixed volume ratios of sample and reagents to other components. Purification or separation of the desired constituent is effected by continuous dialysis and, after appropriate chemical treatment, optical density is measured in a ratio-recording dual-beam colorimeter fitted with a flow cuvette. Samples are introduced by means of a circular rotating sample plate synchronized with the other components of the system. Samples can be analyzed at the rate of 20, 40, or 60 per hour at the discretion of the operator.

The colorimetric method for Terramycin analysis has been automated in our laboratories by use of the AutoAnalyzer system. The system is now in satisfactory routine use for control purposes. Since we are not aware of any so-called manual colorimetric methods that do not require many changes in detail for ultimate adaptation to the AutoAnalyzer system, we propose in this communication to outline the experimental work required in automating a relatively simple colorimetric method by using existing instrumentation.

### *Experimental*

Manual colorimetric determination of Terramycin, as described by Monastero *et al.*, is carried out in several steps. The antibiotic is separated initially from interfering excipients or other material by a suitable analytical separation, usually filtration or liquid-liquid extraction. The solution to be analyzed is adjusted to pH 2 and diluted with 0.01 *N* HCl so that a final 10.0-ml. volume will contain approximately 0.5 mg. Terramycin. Ten milliliters of 0.5 per cent ferric chloride in 0.01 *N* HCl is added, and the mixture is allowed to stand for 10 min. at room temperature for color development. At the end of this period, the optical density at 490  $m\mu$  is measured in a suitable colorimeter

\* Technicon Instruments Corp., Chauncey, N. Y.

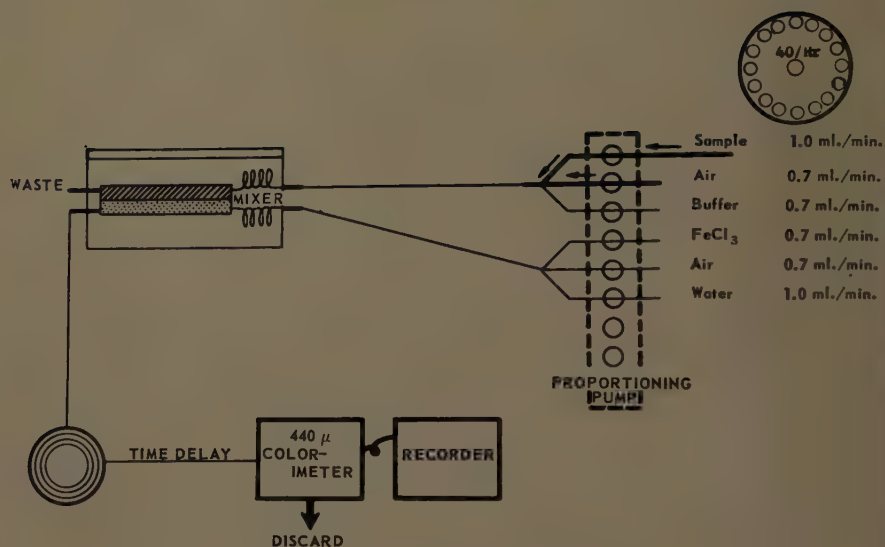


FIGURE 1. Initial generalized flow diagram.

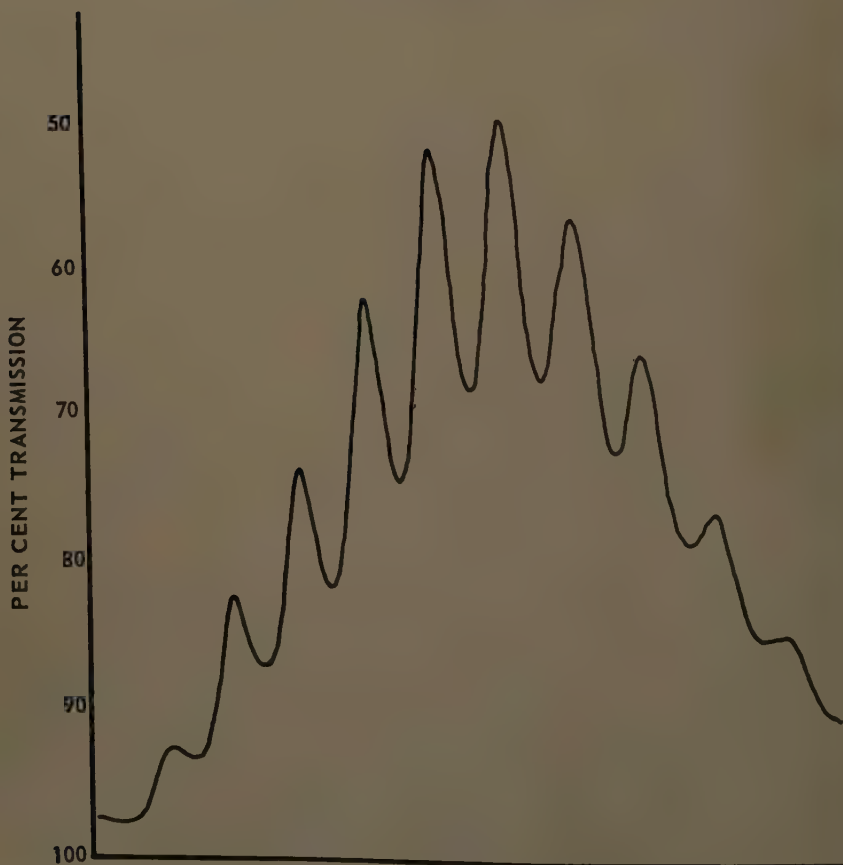


FIGURE 2. Typical response pattern with flow diagram of FIGURE 1.



against a reagent blank. The concentration of Terramycin in the sample is estimated from the appropriate standard curve. Reproducibility of a determination is of the order of  $\pm 2$  per cent. The methods have been applied successfully to tablets, ointments, parenterals, elixirs, and solutions.

Trial analyses with the AutoAnalyzer were designed to carry out the method essentially as outlined above. Such factors as volume ratios, reagents, *pH*, and

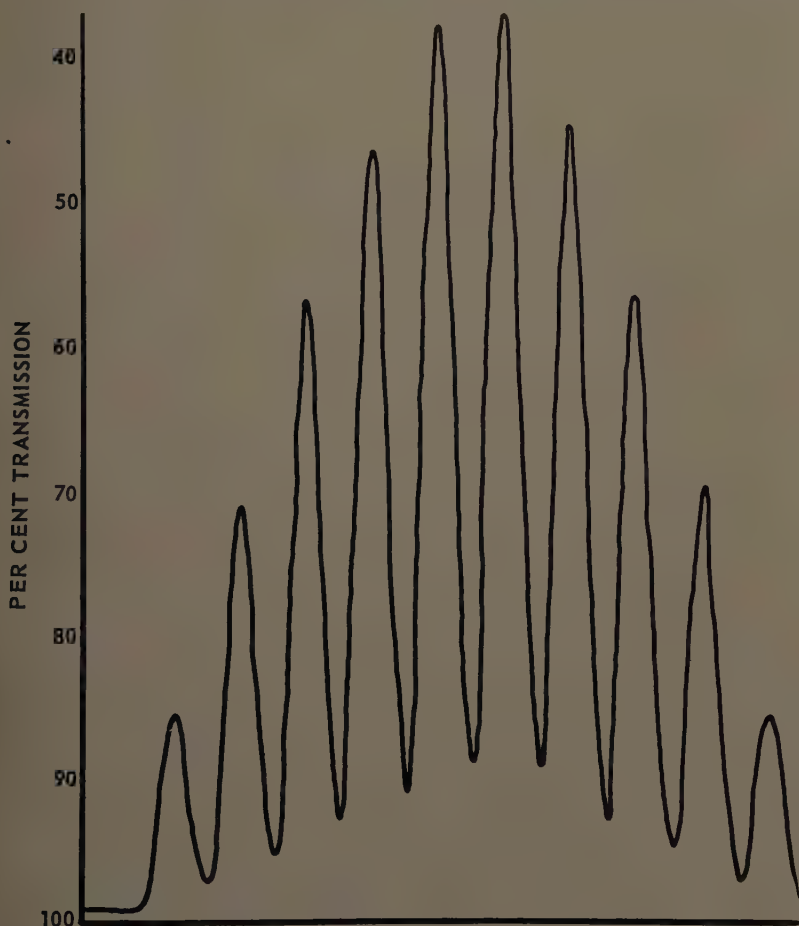


FIGURE 3. Response pattern obtained with manifold in TABLE 1.

timing were not altered substantially. However, a spectrophotometric study of the colored Terramycin- $\text{Fe}^{3+}$  complex revealed a higher absorption peak at about  $420 \text{ m}\mu$ ; hence a filter in this range was used in our work. The earlier generalized manifolds (the series of tubing in the pump which determine volume ratios and directions of flow) and instrument flow sheets are seen in FIGURE 1. The data to the right of the proportioning pump refer to the volume delivered in the particular section of tubing. The sample is introduced from the sampling plate (40 samples per hour), segmented by air, and diluted with

0.01 N HCl, which adjusts the pH to 2. The diluted sample is passed into the dialyzer and dialyzed against a concurrent stream of the ferric chloride reagent previously diluted and segmented by air. During this step, a portion of the Terramycin diffuses across the membrane (du Pont Cellophane No. 300-PT-62)

TABLE 1  
MANIFOLD FOR AQUEOUS TERRAMYCIN STANDARDS\*

Constituent	Relative flow rates (vol./min.)
Sample	1.5
Sample-air	0.5
Sample-buffer	1.0
FeCl <sub>3</sub>	1.0
Dialysate-air	1.0
Dialysate-buffer	1.0

\* The response obtained with the use of this manifold and the flow diagram of FIGURE 1 is given in FIGURE 3.

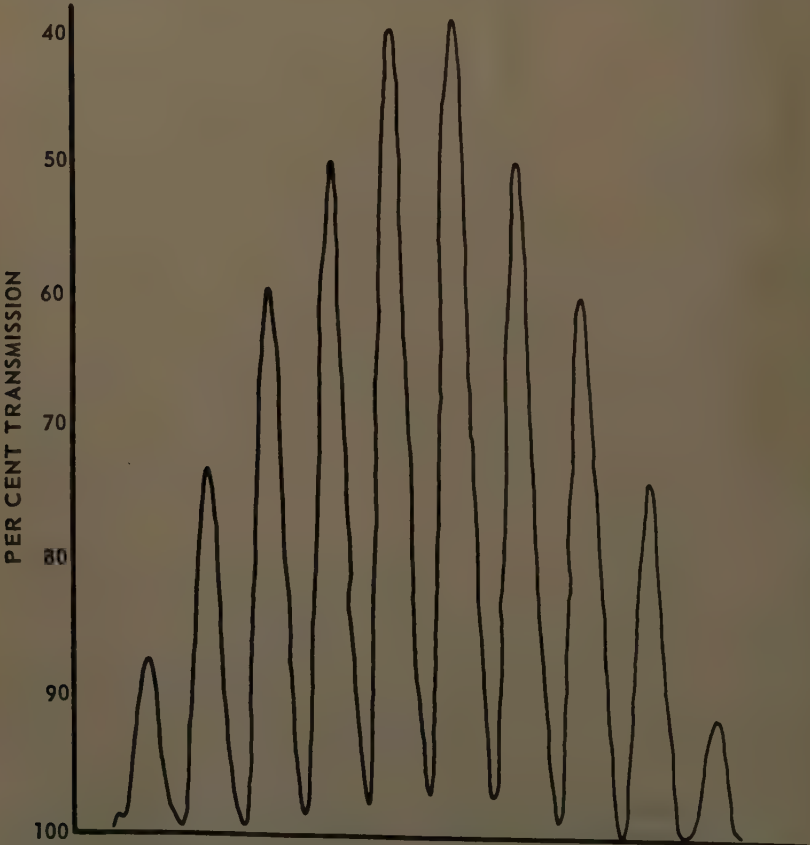


FIGURE 4. Response pattern obtained with flow diagram including dialysis against buffer.

and color development begins; nondialyzable materials including excipients are discarded. The reagent stream is directed into a 5-min. time-delay coil to allow for full color development. After passage through the coil, the optical density of the stream is measured in the colorimeter and recorded. A 440-m $\mu$  filter and room-temperature dialyzer were used during the early phase of this

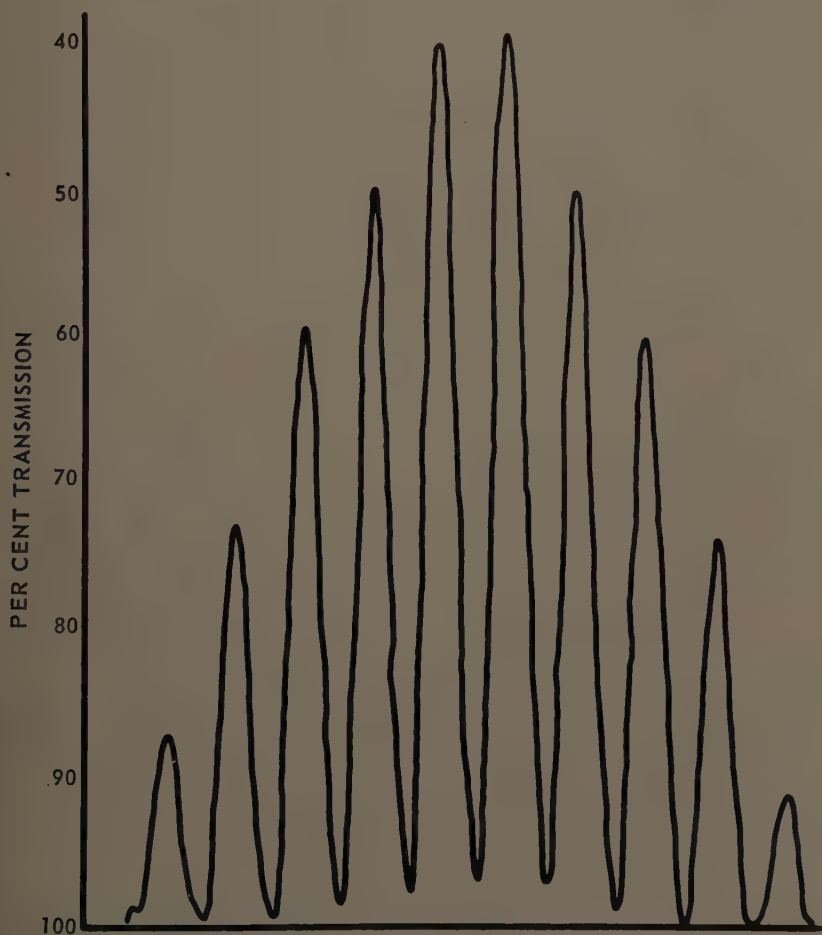


FIGURE 5. Response pattern with flow diagram of FIGURE 6.

work, until a 420-m $\mu$  filter and constant-temperature dialyzer unit were obtained. A typical response of a series of aqueous standards of Terramycin is shown in FIGURE 2. It may be seen that there is contamination between successive samples and, hence, no return to the base line. In addition, the sensitivity is entirely unsatisfactory. The next series of experiments was designed to eliminate these undesirable features. The volume ratios were varied in factorial sequence by changing the combinations of tubing diameters. The other parts of the flow process were held constant. FIGURE 3 shows the best

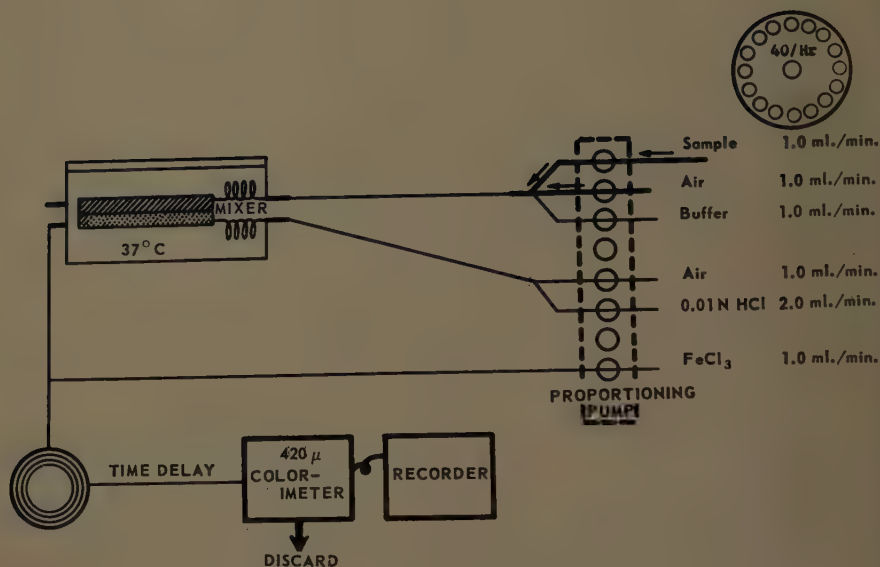


FIGURE 6. Flow diagram of present system.

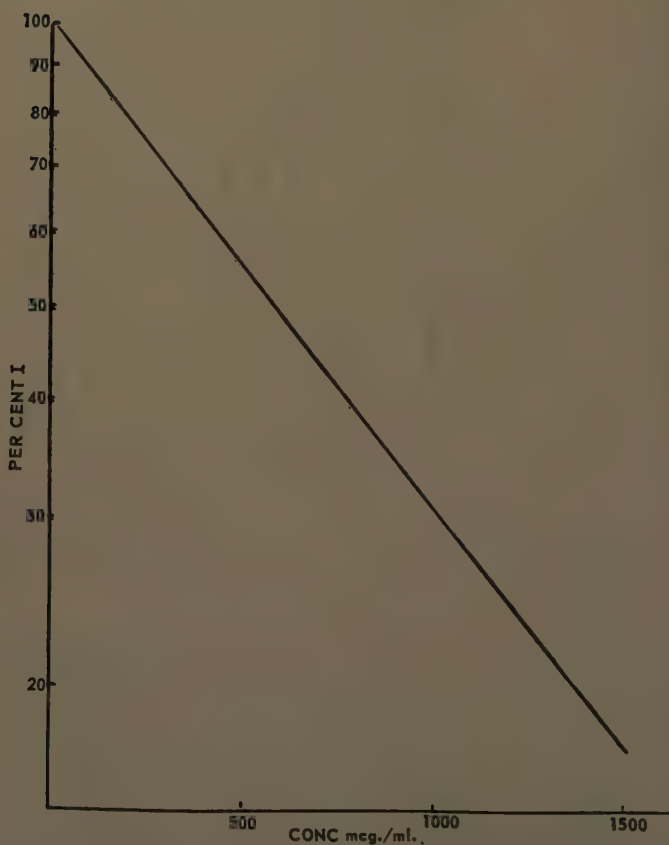


FIGURE 7. Typical standard curve for Terramycin; flow diagram of FIGURE 6.



response pattern obtained from this series of experiments; the manifold is given in TABLE 1. During the course of these experiments the desirability of a number of changes became obvious. The separation of samples with distilled water washes decreased the contamination from sample to sample; this practice was adopted as a standard procedure. Since pH is critical for reproducibility, Clark and Lubs buffer (pH 2.0) was used in place of acid and water. Apparently, when new cellophane membranes are used, a considerable amount of Terramycin is adsorbed on the surface. Subsequently, a small amount is desorbed and will appear in the samples. This phenomenon can be overcome by running a standard solution through the membranes for several hours before actual samples are introduced, or by equilibrating the membranes in a Terramycin solution for several hours before insertion into the dialyzer unit. It can be seen in FIGURE 3 that sample cross-contamination thus was reduced and sensitivity was increased. The response pattern, however, was still not satisfactory.

In another series of experiments, a number of changes were made in the flow diagram. When the buffered sample was dialyzed against buffer and ferric chloride reagent was introduced after the dialysis step, a considerable reduction in cross-contamination was noted. The incorporation of this change with essentially the manifold given in TABLE 1 resulted in the response pattern shown in FIGURE 4. Additional trial revealed that dialysis against 0.01 *N* HCl instead of buffer further reduced cross-contamination. While these two changes in the flow diagram were held constant, the volume ratios in the manifold again were changed in factorial sequence. FIGURE 5 shows the best pattern obtained from this study, and FIGURE 6 sets forth the entire flow diagram. A typical standard curve is seen in FIGURE 7. This curve has been reproduced over a period of several weeks within an error of  $\pm 1$  per cent.

Although this flow diagram does not give complete return to the base line in all cases, it has been found to be a practical scheme for the routine analysis of a wide variety of samples containing Terramycin. Results on samples are reproducible within  $\pm 2$  per cent, and no curvature or nonparallel response between standards and samples has been encountered. The method has been applied satisfactorily to many hundreds of different pharmaceutical formulations.

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# CONTINUOUS AND AUTOMATIC ANALYSIS OF PROCESS STREAMS BY GAS CHROMATOGRAPHY

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## *Introduction*

Rising labor and production costs have dictated that chemical and petroleum plants be operated at peak efficiencies. During the last decade conventional instruments such as pressure and flow controllers have been greatly improved, and various single-component composition analyzers have been introduced to the process industries to help improve process control and product quality and yield.

Most process streams consist of more than two chemical components and, while a single-component analyzer is useful in monitoring the quality of either the product or one of the impurities, optimal process performance demands more complete and more frequent knowledge of the stream composition. The analyzer capable of performing multicomponent analyses of process streams is the automatic gas chromatograph. Although it has been available for only a few years, it has proved to be a most useful and versatile analyzer.

The automatic gas chromatograph has gained wide acceptance in the process industry, and its reliability and performance are surpassed only by the very simplest of composition analyzers.

## *Design Considerations of the Automatic Gas Chromatograph*

Gas chromatography differs from most methods of instrumental analysis in that it performs a quantitative separation of a mixture prior to the detection and measurement of the components of the mixture. The separation is not performed instantaneously; the time required varies with the nature of the sample and the parameters of the gas chromatograph. The separations performed by the gas chromatograph result in simple binary mixtures, each containing a common component not present in the original mixture. Detection and measurement of the separated components are relatively simple, since they contain no interfering components, and all components may be measured as long as they fall within the sensitivity of the detector.

Unlike other methods of instrumental analysis, the gas chromatograph does not lose its ability to measure all the desired components of a mixture when the technique is automated. Instruments employing such methods as infrared analysis and refractometry usually become single-component analyzers when automated. On the other hand, the automation of the gas chromatograph results in a discontinuous or cyclical analyzer, while other analyzers are continuous. Because the analysis of the automatic gas chromatograph is cyclical, all the functions of the analyzer must be programmed, and all are important factors in the design considerations.

The automatic gas chromatograph must perform the following functions automatically: (1) introduce a measured volume of sample into the chromatographic column; (2) perform the desired separation while the sample travels

through the column; (3) detect and measure the components of the sample as they elute from the column; (4) record only components of interest; (5) standardize (or zero) the chromatographic detector; and (6) repeat the above steps automatically and reproducibly.

Perhaps the most important function of the automatic gas chromatograph is that it perform its analyses reproducibly. To ensure this, the following parameters of the analyzer must be closely controlled: temperature of the chromatographic column, column efficiency, sample size, carrier flow rate, and



FIGURE 1. Reproduced by permission of the Instrument Society of America.

temperature and pressure in the detector. In the design of the analyzer, these variables must be considered carefully if the analyzer is to perform reliably in a plant that is subject to varying conditions of temperature and humidity.

#### *Descriptions of the Automatic Gas Chromatograph*

The process gas chromatograph is normally divided into three major assemblies: the sensing unit, the programmer, and the recorder (FIGURE 1). To meet the requirements of a process installation, the sensing unit or analyzer is enclosed in a housing suitable for Class I, Group D, Division I, hazardous locations, and the programmer and recorder are enclosed in housings suitable for Class I, Group D, Division II, hazardous locations.

*Sensing unit.* The sensing unit contains the chromatographic column, de-

• tector, sampling valve, carrier gas flow controller, and carrier and sample gas heat exchange coils. Since each of these components is affected to some degree by temperature changes, all are enclosed in a constant-temperature bath. One system of temperature control is shown in FIGURE 2. Components requiring



FIGURE 2. Reproduced by permission of the Instrument Society of America.

the most precise temperature control are all in contact with an aluminum mandrel. Heat is supplied by electric heaters, and the temperature sensor is a mercury-in-glass thermometer with electrical contacts. The lower portion of the analyzer contains the carrier gas flow controller and all electrical components. Since these components deteriorate rapidly at high temperatures, this portion of the analyzer is thermostated separately at a temperature lower than that of the mandrel.



Another system of temperature control employs a proportional controller that regulates the ratio of hot and cold air blown over the components of the analyzer. Either system is capable of maintaining the desired temperature within  $\pm 0.2^\circ \text{C}$ . Ambient temperatures may vary from  $-10$  to  $115^\circ \text{F}$ . without affecting the analyzer when it is set for  $50^\circ \text{C}$ . The upper limit of operation varies between  $150$  and  $200^\circ \text{C}$ ., depending on the design of the analyzer.

After the components have been separated and have emerged from the chromatographic column, their respective concentrations in the effluent stream must be measured. In the automatic gas chromatograph either thermal conductivity or catalytic combustion detectors are employed.

Thermal conductivity detectors employ either thermistors or wire filaments. As they are extremely sensitive to temperature changes, they must be located in the thermostated bath. The design of the detector chambers is very critical. These chambers must be designed for maximum sensitivity to small concentrations with minimal sensitivity to small flow and pressure changes. Aside from concentration differences, the response and sensitivity of the detectors are proportional to the difference in thermal conductivity between the carrier gas and the component. Helium or hydrogen normally are used because they offer the largest thermal conductivity differences between themselves and the components.

In the catalytic combustion detector the separated components are burned catalytically. The carrier gas must be either air or oxygen. In this detector the filaments initiate burning and measure heats of combustion of the components.

Both types of detectors have been designed to be as stable as possible but, because they tend to age and change their characteristics with time and because thermal drifts cannot be eliminated completely, it has been found necessary to include some method of automatically zeroing the detector circuit once during each analysis cycle.

To ensure the reproducibility of the analysis, the volume of sample introduced at the start of each analysis cycle must be made constant. This is accomplished by trapping the sample gas in a fixed volume of tubing and by controlling the temperature and pressure of the sample gas. It is important to note that size of the sample is quite important, since it is very easy to saturate the detector and thus effect a poor peak resolution.

The sample volume, chromatographic columns, and detector are usually linked together by the sample injection valve. Sample injection valves are either rotary or linear in operation and, normally, are constructed of stainless steel and Teflon, to eliminate the need for lubrication between either the stator and rotor or the stem and block.

A rotary valve is schematically illustrated in FIGURE 3. In the upper portion of the diagram the valve is in the clockwise position. The sample flows through the sample volume and then to the sample vent. Carrier gas flows through the chromatographic column to the detector and then to the vent. A portion of the carrier gas is diverted to the reference detector and then to the vent.

At the start of a new analysis cycle the valve is rotated to the counterclockwise position. The sample now bypasses the sample volume and goes directly

to the vent. Carrier gas sweeps out the sample volume and then flows to the column. The valve remains in this position until shortly before the start of a new cycle, when it is rotated back to the clockwise position. Fresh sample enters the sample volume, and the cycle starts anew.

*Programmer and recorder.* The programmer controls all functions of the automatic chromatograph (FIGURE 4). Its heart is a timer, and the sequence of its operation is visible at all times to the operator through the master timing dial (FIGURE 5).

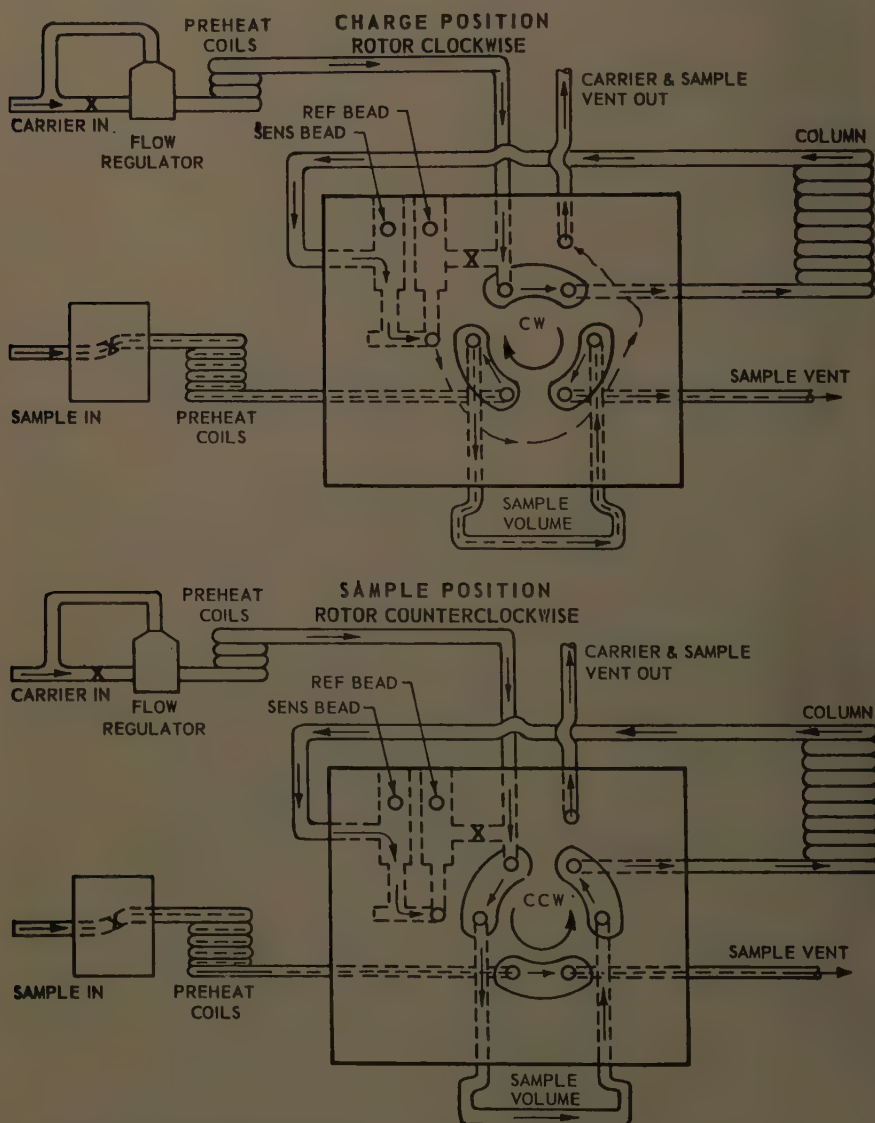


FIGURE 3. Flow schematics for two positions of the sampling valve. Reproduced by permission of the Instrument Society of America.

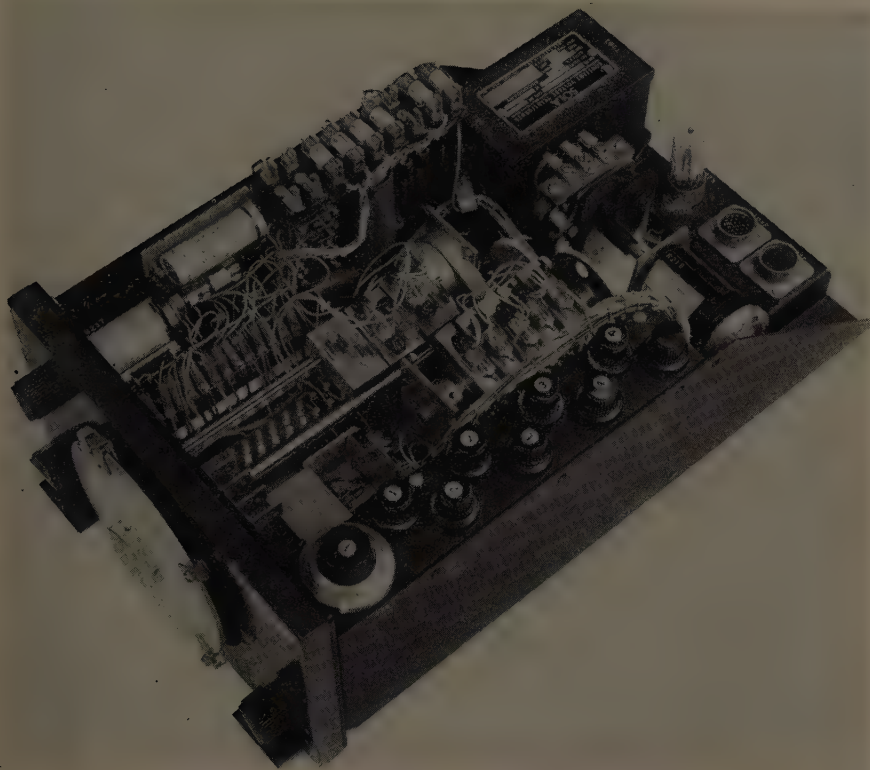


FIGURE 4. Multistream dual range programmer.



FIGURE 5. Reproduced by permission of the Instrument Society of America.

The following are the functions and sequence of operation of the programmer: (1) introduce the sample into the chromatographic column; (2) simultaneously close the circuit between the sensing unit and the programmer and feed the signal through the appropriate attenuator when the desired component enters the detector; (3) open the circuit between the sensing unit and programmer just before the last of the component leaves the detector; (4) advance the recorder chart a small distance to separate the individual components; (5) repeat steps 2 through 4 for each component; (6) activate the valve to allow fresh sample to enter the sample loop; (7) activate automatic bridge zeroing at some point in the cycle when no components are passing through the detector; and

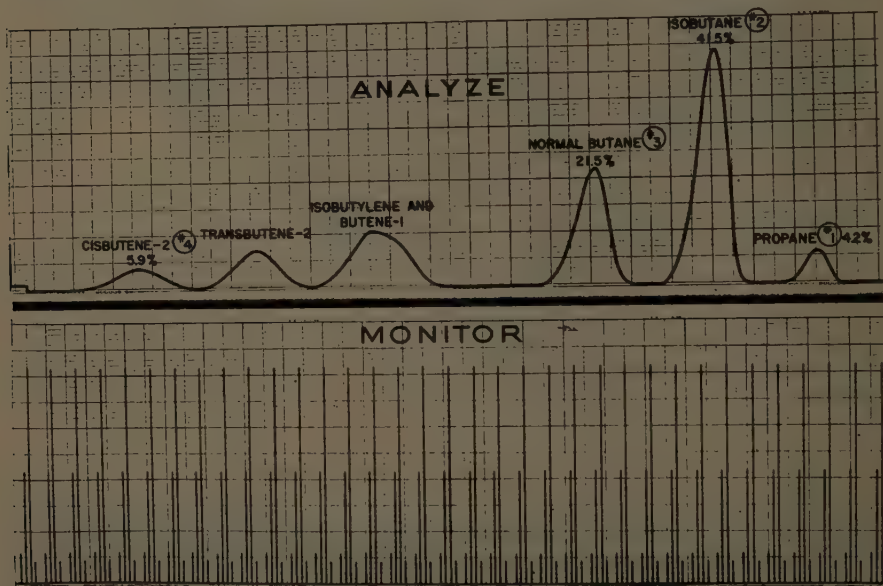


FIGURE 6. Model 184 fractogram: a mode of chart presentation showing quantitative analysis of components from typical refinery stream. Per cent concentrations of four components selected from fractogram are shown directly on bar chart. Reproduced by permission of the Instrument Society of America.

(8) move the recorder chart an additional amount between the first and last components to identify the start of a new analysis.

FIGURE 6 shows a typical chromatographic analysis of a gas mixture. The bottom half of the figure shows in bar graph form the continuous analysis of four components of the same mixture, with each component properly attenuated to reflect its composition in the mixture. The bar graph form of analysis has found wide acceptance, since it is easy to read and conserves chart paper. Individual attenuation of the components is necessary because each component has a different thermal conductivity and band width. No two components normally will produce the same peak signal for equal concentration in the helium carrier.

In addition to providing automatic operation, the programmer must provide means for facilitating calibration and periodically checking the timing setup.



One mode of operation particularly useful for component identification and initial calibration is the fractogram operation. In this operation the timer motor is dead, and the entire output of the detector is channeled through one attenuator. This mode of operation completely duplicates the operation of a laboratory chromatograph. A second mode of operation is the check timing operation. This operation is the same as the bar graph operation except that the chart is allowed to run continuously. This allows the operator to check the performance of the column and the timing mechanism without disturbing the analysis.

A third mode of operation is used only when the chromatograph is being calibrated. In this operation, the attenuators are connected one by one to a test signal that can be varied to duplicate a detector signal. For instance, the peak of a component might read 32 when it should read 30 chart divisions. The test signal is switched to the attenuator in question and adjusted until the recorder pen is at 32. The attenuator setting is then changed to give a reading of 30, thereby correcting the calibration of the analyzer for that component.

The programmer described above is of the simplest variety. Many variations exist. Programmers are available that will monitor from 4 to 8 components in a stream and alternately sample as many different streams as the user desires. Of course, the more streams sampled, the lower will be the sampling frequency of any one stream. All manufacturers will modify their programmers to meet unusual applications.

### *Technology of Chromatographic Columns*

The chromatographic column is the heart of the sensing unit. Many factors govern its proper selection, and it is not uncommon to find that different columns must be used for comparable analyses of similar streams.

Chromatographic columns are generally of two types: adsorption and partition. Adsorption columns contain such materials as activated silica gel, alumina, charcoal, or molecular sieve. Partition columns contain partition agents—liquids not volatile at high temperatures—on solid supports.

Adsorption columns have an advantage over most partition columns in that they will not deteriorate at high operating temperatures. Their chief disadvantage is that they tend to adsorb certain materials to equilibrium saturation. This makes a portion of the adsorbent inactive. The adsorbed materials normally are found as impurities in the carrier gas. As the concentrations of these impurities in the carrier gas change, the equilibrium saturation and the amount of inactive surface in the adsorbent change, and the elution times of all the components change. This situation may be alleviated somewhat by lightly coating the adsorbent with a partitioning agent. Adsorption columns are generally useful for the light gases and hydrocarbons of low molecular weight.

Partition columns are more generally applicable than adsorption columns because of their ability to separate many classes of organic compounds over wide ranges of molecular weights and boiling points. This is not to infer that any partition agent will do; different agents will perform different separations, and the proper agent must be chosen for the desired separation.

Besides the ability to perform the desired separation, the choice of partition

agent is generally limited by two factors: temperature stability and inertness. The minimum temperature of the automatic chromatograph is dictated by the prevailing ambient temperature. The upper limit of temperature is determined by the design of the analyzer; the operating temperature is governed by the requirement that the sample be in the gaseous state while it is in the analyzer. Many partition agents that are suitable at room temperature or in laboratory chromatographs are unsuitable for continuous use in the automatic chromatograph because of their high volatilities at elevated temperatures. Others that are not so volatile at elevated temperatures suffer a decrease in efficiency of separation. The last may be partly compensated for by increasing the column length, but it is best to find a new agent that is relatively non-volatile at the elevated temperature and that will perform the desired separation.

Very often no one column will perform the desired separation. At other times some one component in the sample, such as water, will react irreversibly with the partition agent. In many instances difficult separations may be achieved by using two or more columns that can be switched selectively into or out of the column train. Where a component of the sample is apt to react with the partition agent, frequently a guard column, which will greatly retard the flow of the contaminant relative to the other components in the mixture, may be placed in series with the main column. When the desired components have left the guard column and entered the separatory column, the separation proceeds as usual, and the guard column is backflushed to remove the contaminant from the system.

Thus far columns have been discussed only with regard to their ability to perform a given separation. That is not enough. The time required to perform the analysis is most important for, if the analysis takes too long, the automatic chromatograph is of no more value than the laboratory chromatograph.

In most instances in which it is not possible to achieve a desired separation in a reasonable time with one column, it is possible to do so by optimizing carrier gas flow rate and analyzer temperature and by using varying lengths of different chromatograph columns either in series or in an automatically programmed sequence. Most automatic analyses require from 5 to 15 min.

To utilize two or more columns in a specific sequence of operation or to be able to backflush one or more columns, the valving inside the sensing unit must be altered. With the rotary valve this is accomplished by increasing the number of ports and channels. With linear valves it is normally accomplished by placing additional valves in the column train.

*Sampling systems.* Although the process vapor fractometer has been discussed in considerable detail, the sampling system that accompanies it must not be slighted. The requirements for the sample, prior to its entry into the analyzer, are that it be clean and entirely in the vapor state, and that it not exceed the safe operating pressure of the sampling valve. A typical multi-component sampling system is illustrated in FIGURE 7. The system illustrates the application of both liquid and gaseous streams in the same system.

All the necessary components for pressure regulation, sample vaporization, stream switching, temperature control, and sample flow control are illustrated.

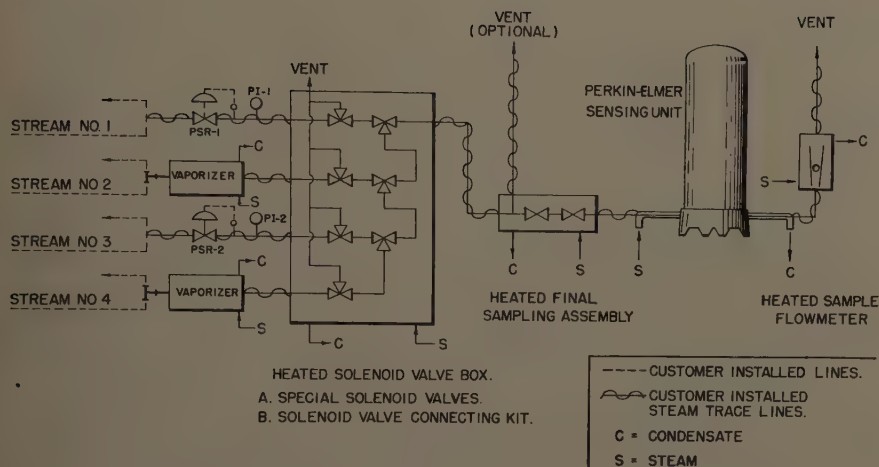


FIGURE 7. Reproduced by permission of the Instrument Society of America.

TABLE 1  
APPLICATIONS OF AUTOMATIC GAS CHROMATOGRAPHS;  
PETROLEUM AND PETROCHEMICAL INDUSTRY

Process and units	Analysis
Natural gasoline plant	
Absorber	Methane, ethane, CO <sub>2</sub> , propane
De-ethanizer	Methane, ethane, propane
Depropanizer	Ethane, propane, propylene, isobutane
Debutanizer	Propane, isobutane, <i>n</i> -butane, isopentane
Butane splitter	Isobutane, <i>n</i> -butane, isopentane, <i>n</i> -pentane
Butane isomerization	
Isomerization product	Propane, isobutane, <i>n</i> -butane
Butane splitter	
overhead	Propane, isobutane, <i>n</i> -butane
bottoms	Isobutane, <i>n</i> -butane, isopentane
Alkylation	
Feed	Propane, isobutane, <i>n</i> -butane, butylenes
Debutanizer	Propane, isobutane, <i>n</i> -butane, isopentane
Depropanizer	
overhead	Ethane, propane, propylene, isobutane
bottoms	Propane, isobutane, <i>n</i> -butane, isopentane
De-isobutanizer	
overhead	Propane, isobutane, <i>n</i> -butane, isopentane
bottoms	Propane, isobutane, <i>n</i> -butane, isopentane
Ethylene	
Absorber	H <sub>2</sub> , CO, CO <sub>2</sub> , methane, ethane, ethylene
De-ethanizer	CO, CO <sub>2</sub> , methane, ethane, ethylene
Ethane splitter	Ethane, ethylene, propane
Depropanizer	Ethane, ethylene, propane, isobutane
Polymerization	
Reactor feed	Methane, ethane, propane, propylene, isobutane
Splitter	Ethane, propane, propylene, isobutane
Depropanizer	Ethane, propane, propylene, isobutane
Inorganic chemistry	
Ammonia synthesis	CO, A, N <sub>2</sub> , H <sub>2</sub> , methane, ammonia
Sulfur recovery	O <sub>2</sub> , N <sub>2</sub> , H <sub>2</sub> S, SO <sub>2</sub>
Organic chemistry	
Alcohol plants	Water, methanol, ethanol, propanol, acetone
Benzene, toluene, xylene plants	Hexane, benzene, toluene, xylenes
Freon production	Mixed Freons

The vaporizer usually is a combination vaporizer and pressure regulator. Pressure regulation is obtained by using various lengths of capillary tubing in the vaporizer. The stream-switching assembly employs the block and bleed system. This system is recommended because it minimizes the possibility of stream mixing, in the event that some of the valves leak.

All interconnecting lines and vents should be steam-traced and insulated if there is any possibility that the samples may condense at ambient temperatures. All sample runs between the process stream and the analyzer should be minimized to reduce sample lag. At any rate, the lag time in the sampling system should not exceed the analysis time.

Although they are not shown here, sampling systems may contain water separators, chemical scrubbers, and other special equipment. Except for these last accessories, most chromatograph manufacturers supply all the necessary components of the sampling system and provide start-up service, if requested.

### *Applications of the Automatic Gas Chromatograph*

Automatic gas chromatographs are now employed widely in both monitoring and control applications. In monitoring applications the analyzer is equipped with a recorder, and the data presentation is generally in bar-graph form, as already described. In control applications the programmer and recorder must be modified so that pneumatic or electronic signals, which are representative of the concentrations of one or more components of the stream being analyzed, are transmitted continuously.

Although the petroleum and petrochemical industries have been the major users of automatic gas chromatographs, the instrument is now employed in all phases of the process industry. Its use in the organic and inorganic chemical industries has been slow in developing. The reason is not lack of interest or applications. In the field of organic chemistry, chromatographic columns are generally available but, frequently, either the analysis times are too long or the analyzers would be required to operate at temperatures outside their design limits. In the field of inorganic chemistry, chromatographic columns have not been generally available.

In TABLE 1 are listed typical applications of the automatic gas chromatograph. The table lists the major process unit, the specific applications, and the usual chromatographic analysis.

### *The Future of Automatic Gas Chromatography*

The automatic gas chromatograph has proved itself a reliable instrument for process control and control of product quality. Although the cyclic nature of the analyzer cannot be circumvented, it should not be long before analysis cycles of 1 min. or less are obtained. Industry is recognizing that the "four horsemen"—flow, pressure, temperature, and level controls—are not the ultimate in process control and, with the advent of fully automated processes, the automatic gas chromatograph surely will be an important member of the control team.



# AUTOMATED ENZYMATIC ASSAY OF ORGANIC PHOSPHATE PESTICIDE RESIDUES

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The development in recent years of a host of complex and often highly toxic agricultural chemicals has presented tremendous problems of use and residue control to both manufacturers and governmental regulatory bodies. Among these compounds, the group of phosphate ester insecticides stands apart, by virtue of its principal action, the *in vivo* inhibition or inactivation of the enzyme acetylcholinesterase (ChE).

Recently several investigators have proposed variations<sup>1,4,6,10</sup> of a bioassay procedure for determining residues of these compounds in plant material; these methods are based on measurement of the inhibition of ChE by the insecticide present. I have discussed elsewhere in this monograph the automation of ChE-activity determination with the use of the AutoAnalyzer,\* a system for automatic chemical analysis.<sup>8</sup>

## *Experimental*

*Apparatus.* The several modules of the system are described adequately elsewhere.<sup>8,9</sup> For the ChE bioassay technique the following modifications, illustrated in FIGURES 1 and 2, are required:

(1) The sampler plate has not one but two concentric sets of holes for the sample cups and a double sampling pick-up crook so that two samples may be pumped simultaneously.

(2) The 37° C. heating bath module is fitted with two coils, one of standard size and one of a larger bore to provide for a longer-than-normal delay in the incubation bath.

(3) Two proportioning pumps are required.

*Reagents.* The buffer consists of sodium barbital (0.4000 gm.), potassium dihydrogen phosphate (0.0400 gm.), and sodium chloride (12.000 gm.) dissolved up to 1l. in distilled water. Dilutions (1:4) are made with distilled water.

Purified crystalline bovine erythrocyte acetylcholinesterase, 20,000 U./vial,† is dissolved in 10 ml. distilled water. Portions of this stock solution are diluted to 100 U./ml. for use as required.

Since proteinaceous material is removed from the system in the continuous dialysis stage, other sources of ChE, such as horse serum or bee brain, may be used as substitutes for the purified material if desired.

For the analysis, 0.05 ml. saturated bromine water is diluted to 100 ml., and the following substances are each dissolved and made up to 1l. with water: acetylcholine iodide (20.00 gm.), phenol red WS‡ (0.225 gm.), and sodium chloride (100 gm.).

*Standards.* One hundred mg. of the insecticide to be analyzed is dissolved

\* Technicon Instruments Corp., Chauncey, N. Y.

† Winthrop Laboratories, Special Chemicals Dept., New York, N. Y.

‡ Hartmann-Leddon Co., Philadelphia, Pa.

in 30 to 40 ml. ethanol and diluted to 100 ml. with distilled water. Aliquots of this stock solution are then diluted with water to give the required range of insecticide content in a series of standard solutions.

*Preparation of samples.* Limitations imposed by the continuous-flow cuvette and by the polyvinyl chloride tubing that links the instrumental components



FIGURE 1. Automatic system for determining ChE inhibition, showing, from left to right, recorder, flow colorimeter, continuous dialyzer, heating bath with two coils, two pumps, reagents, and sampler.



FIGURE 2. Detail of the sampling, mixing, and pumping system used in this analysis.

require that the solvent in which the insecticides are stripped from the plant material be water-miscible. If the stripping material is not miscible with water, it is necessary to evaporate the solvent and then redissolve the material in alcohol for analysis. In general, chromogenic plant material in the extract will be removed by dialysis and therefore will not interfere with the colorimetric system.

*Procedure.* The instrumental system is arranged according to the flow dia-

gram in FIGURE 3. Due to the dynamics of the continuous-flow system and the fact that ChE is the subject of the analysis, it is not possible to pump ChE as a steady-state reagent and obtain a readily understood curve for each sample. Accordingly, the ChE and the insecticide sample are placed opposite each other in the sample plate, and their respective sample lines are adjusted in length so that the two samples reach the double mixing coil simultaneously. This results in a single peak on the recorder for each sample pair, the height of the peak being governed by the uninhibited remainder of the standard ChE solution after incubation with the insecticide. A no-inhibition peak is obtained, of course, by pairing the standard ChE solution with water instead of with insecticide. To prevent trace contamination or carry-over—that is, an insoluble deposit left in the sample line—a wash of 20 to 30 per cent alcohol in water is used between samples.

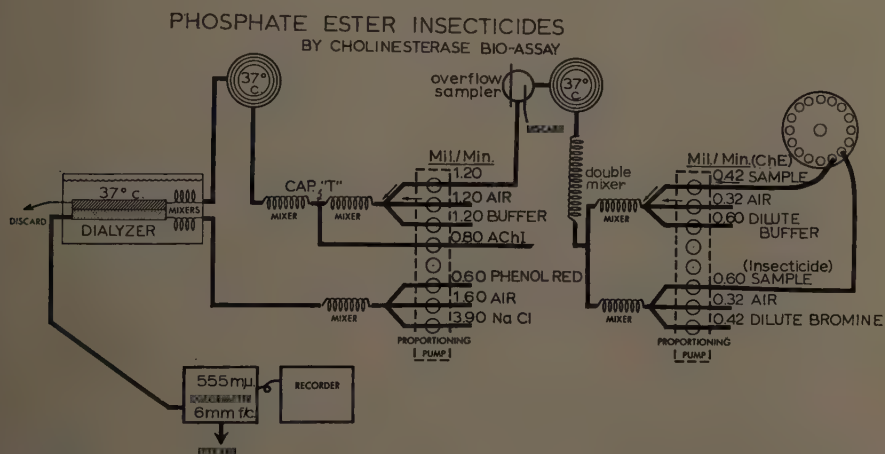


FIGURE 3. Diagrammatic representation of the sample and reagent flow through the analysis system.

After the colorimeter has been adjusted to read 100 per cent transmittance with water flowing through the system, the reagent lines are placed in their respective solutions and the sampler is started rotating so that the samples enter the system in sequence. Sampling can be set at a rate of either 20 or 40 per hour, that is, 10 or 20 analyses per hour plus washes between samples. The time required for the first sample to flow through the entire system is 30 min. Samples are introduced at 3- or 6-min. intervals, depending on the selected sampling rate.

### Discussion

Standards for this test may be prepared from the specific insecticide used on the plants being tested. However, for screening to determine whether any insecticide of the group to which it belongs is present, it would seem much simpler to use standards prepared from a commonly used insecticide such as parathion (O,O-diethyl-O-*p*-nitrophenyl thiophosphate) and relate the results obtained on the ChE in terms of ppm parathion equivalence.

Calibration is accomplished by direct comparison with a semilog plot of the peak values obtained from the standard solutions. To accommodate the wide range of concentration covered, a typical standard curve for parathion is presented in decade form (FIGURE 4). The range of insecticide concentrations shown will be reduced considerably as the sensitivity is enhanced by increasing the concentrations of bromine water.

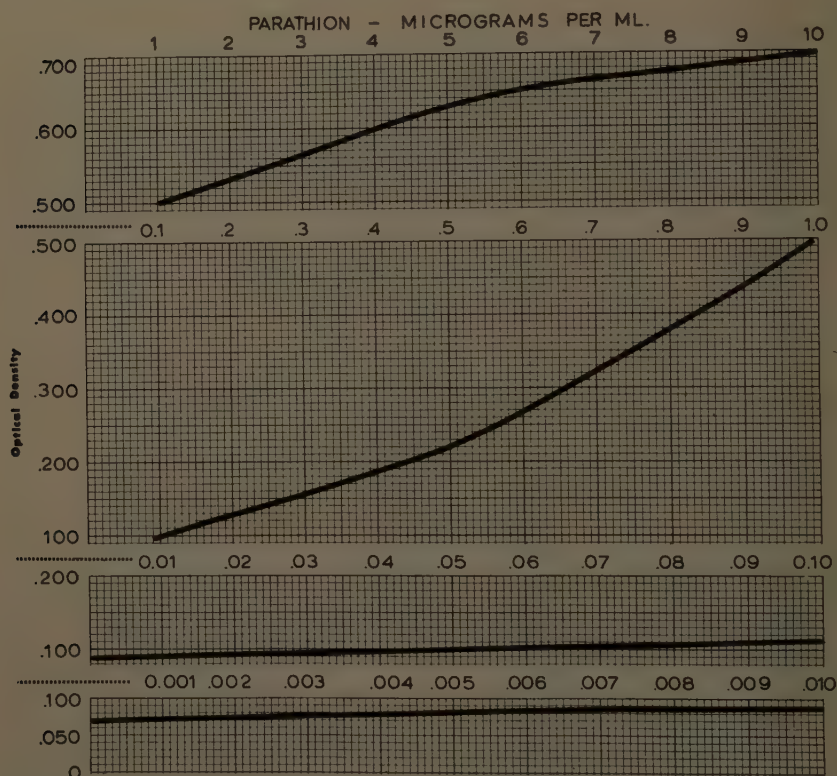


FIGURE 4. Decade-form calibration curve of ChE inhibition by standard solutions of parathion oxidized by 0.04 per cent bromine water.

The bromine-water oxidation of some of these phosphate insecticides to *in vitro* inhibitors has been presented recently by Fallscheer and Cook.<sup>3</sup> It appears, however, that the bromine concentration is more critical in our continuous-flow system than in the batch-type analysis described by these authors. For our method sensitivity increases steadily as the bromine-water concentration is raised from 0.02 to 0.10 per cent, whereas at significantly higher concentrations bromine interferes with the reagent system.

A typical series of standards oxidized by 0.04 per cent bromine water is shown in FIGURE 5. In FIGURE 6 is shown the inhibition of human blood serum ChE by standard parathion solutions without bromine water, as compared with the greatly increased effect due to addition of 0.1 per cent bromine water



to the insecticide prior to incubation with the enzyme. Tracings in FIGURE 7 show the inhibitory effect of four typical insecticides, (1) Thimet: O,O-diethyl-S'-(ethylthiomethyl)phosphorodithioate; (2) parathion; (3) diazinon: O,O-

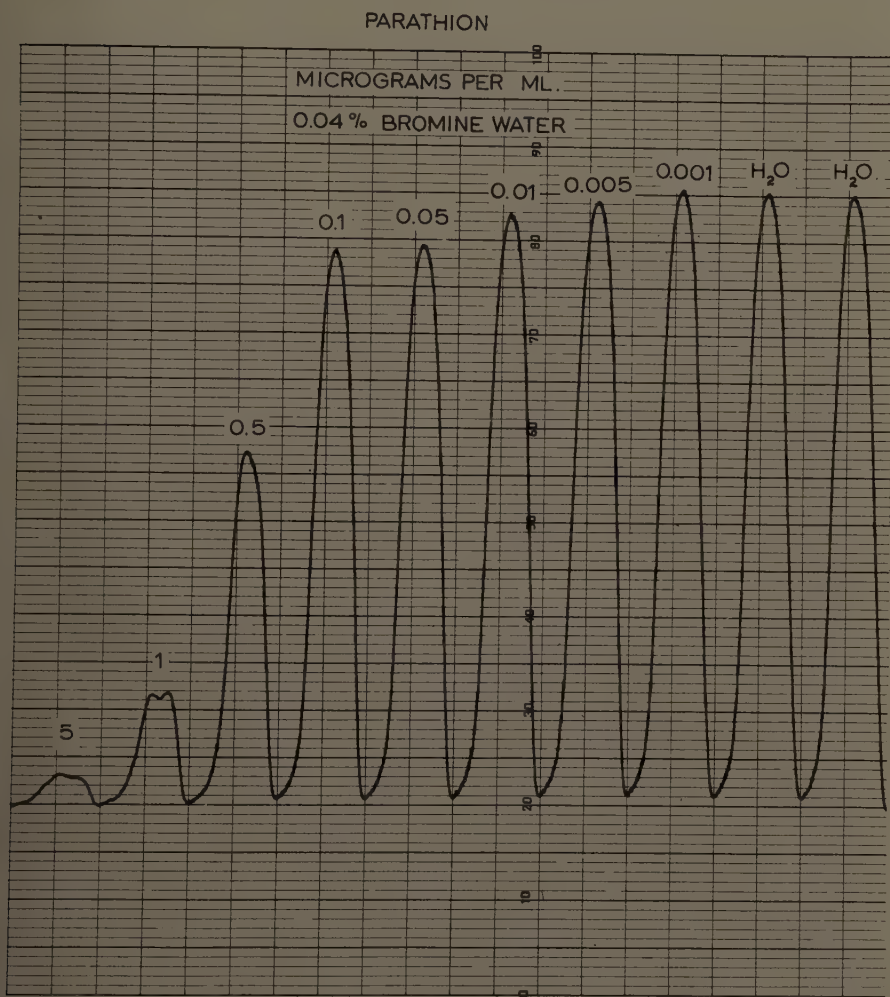


FIGURE 5. Typical recordings of inhibition of ChE by standard parathion solutions with 0.04 per cent bromine water.

diethyl-O-(2-isopropyl-4-methyl-6-pyrimidinyl)thiophosphate; and (4) malathion: S-[1,2-bis(ethoxycarbonyl)ethyl]O,O-dimethylphosphorodithioate. At present this method is particularly useful for screening residues. It is probable that a considerable improvement in specificity could be obtained through a detailed study utilizing different enzyme sources, as suggested by Cook<sup>2</sup> and

Schechter and Hornstein.<sup>7</sup> An excellent preliminary study along these lines has been published by McCaulley and Cook.<sup>5</sup>

### Summary

A method is presented whereby food or plant extracts containing phosphate ester insecticides (with or without the dilute bromine water required to convert

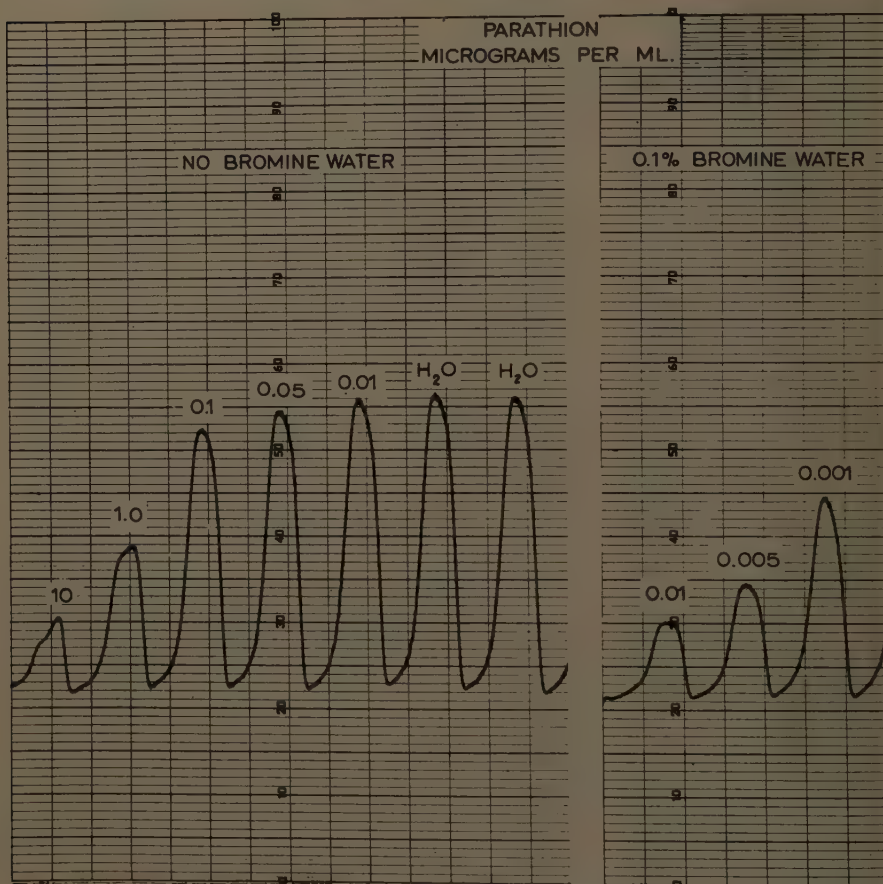


FIGURE 6. Inhibition of ChE by parathion solutions with and without bromine water oxidation of the parathion. The ChE source is pooled human blood serum.

some of these compounds to *in vitro* inhibitors) may be incubated with ChE in a continuous and automatic flow system. The resulting partially inhibited ChE is measured, and the relative inhibition due to the individual insecticide samples is shown as a series of separate peaks on a recorder. The method is particularly suited to screening many samples for organic phosphate insecticide residues because it is possible to perform as many as twenty analyses per hour without operator intervention.

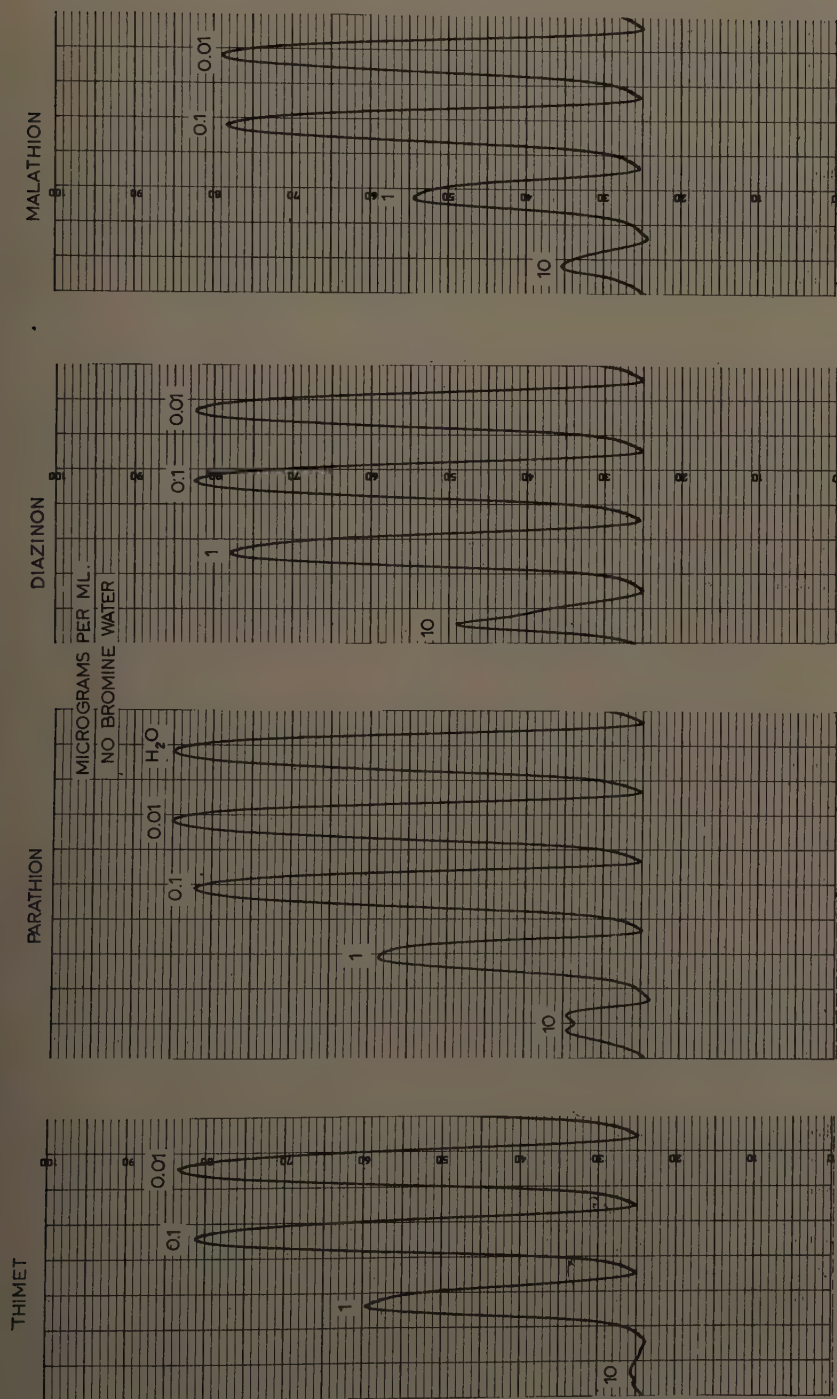


FIGURE 7. *In vitro* inhibition of ChE by standard solutions of four different insecticides.

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# USE OF THE AUTOANALYZER FOR DETERMINATION OF PROTEIN, PHOSPHATE, AND REDUCING SUGARS IN YEAST AND MOLASSES

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The AutoAnalyzer\* is being used at Anheuser-Busch, Inc., to check continuously the quality of bakers' yeast. Specifically, it is employed to determine protein and phosphate content of the yeast as well as protein, phosphate, and sugar content of molasses, the principal nutrient employed in the fermentation procedure.

In order to produce a satisfactory cake of bakers' yeast, its dough-fermentation potential must be controlled carefully. This is done best by controlling the growth of the yeast so that the final protein and phosphate content is held within very narrow ranges. To control yeast growth, it is necessary to determine sugar, protein nitrogen, and phosphate content of the molasses nutrient so that the proper supplemental levels of ammonia and phosphate can be supplied during the yeast propagation cycle. Phosphate and protein content of the yeast are determined as a final check.

Phosphate content of the yeast is used as an index of the nucleic acid level. Past experience has shown that free esterified phosphates such as ATP, which are involved directly with the energy cycle and with the phospholipid fraction, are present at relatively constant levels. If total protein nitrogen and  $P_2O_5$  are determined, nucleic acid content and the globular protein content can be calculated with reasonable accuracy.

## EXPERIMENTAL

The small peristaltic pump, the heart of the AutoAnalyzer, picks up sample and reagent and forces them through nonwetttable Tygon tubing. Since sample and reagents flow continuously, essentially any procedure the analytical chemist employs can be adapted to this system.

The constant-flow characteristic of the pump and the variety of rigidly controlled tubing diameters available make it possible to employ optimum sample-to-reagent ratios for color development. In all cases, colorimetric tests are employed and colorimeter values measured by the photoelectric cell are amplified sufficiently to drive a recording instrument.

### *Analysis of Yeast*

Yeast samples for analysis are taken either from the washed yeast cream from the yeast separators or from the compressed yeast cake produced by high-pressure filtration of the yeast cream. Subsequently, the yeast sample is dried and a standard suspension prepared, or a turbidimetrically standardized dilution is made directly from the fresh sample. The yeast preparation then is handled in the following manner:

*Preliminary digestion.* Yeast (100 to 200 mg.) is mixed with 8 ml. concen-

\* Technicon Instruments Corp., Chauncey, N. Y.

trated sulfuric acid in a Kjeldahl digestion flask. Copper sulfate (100 mg.) and 2 gm. potassium sulfate are added and digestion proceeds for 40 min. The sample is cooled, and water is added to adjust the volume to 115 ml. Aliquots are then presented to the AutoAnalyzer for ammonia and phosphate analysis.

*Protein.* The flow diagram in FIGURE 1 outlines the ammonia determination. The sample is segmented with air and introduced into a stream of 5 *N* NaOH. Tubing diameters are such that 0.6 ml. sample per min. joins a stream of 1.6 ml. NaOH per min., and the stream is segmented with air bubbles introduced at the rate of 0.6 ml./min. After sample and base have been thoroughly mixed

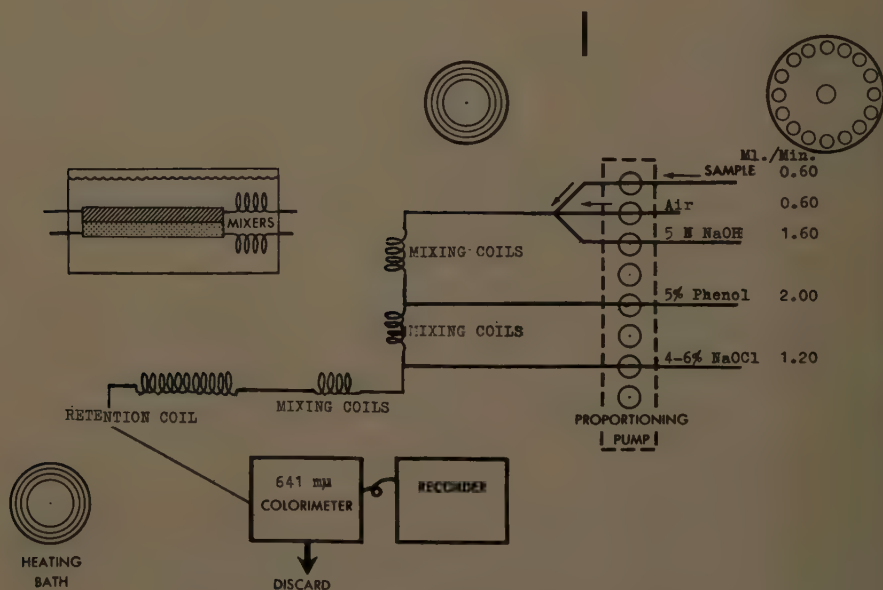


FIGURE 1. Flow diagram for ammonia determination.

in a simple mixing coil, the sample stream is mixed further with 5 per cent phenol flowing at 2 ml./min. The sodium hypochlorite flowing at 1.2 ml./min. then is mixed into the stream. Approximately 3 min. is allowed for maximum color development by passing the mixture through 30 feet of Tygon tubing arranged in a horizontal coil. The sample then enters a flow cuvette with a 4-mm. light path. Color intensity is measured at 641  $m\mu$ , and the results are recorded on arithmetic paper by the recording instrument.

FIGURE 2 shows typical results obtained when digested samples, reference standards, and water blanks are presented to the AutoAnalyzer.

These results and those that follow are taken directly from operating charts in the yeast plant control labs. Standard plots are also presented. The two yeast samples, numbered 49 and 51, come from routine yeast fermentations. For protein analysis, of course, care must be taken to keep the area free from ammonia vapors.

Results from the AutoAnalyzer agree within 3 per cent with results obtained by steam distillation and titration of the ammonia.

**Phosphate.** The method adopted (FIGURE 3) is a modification of the Fiske and Subbarow procedure.<sup>1</sup> It is based on the formation of phosphomolybdic acid, which is then reduced by 1-amino-2-naphthol-4-sulfonic acid. The sample (1.2 ml./min.) and a solution of sulfuric molybdate (2.5 ml./min.) are

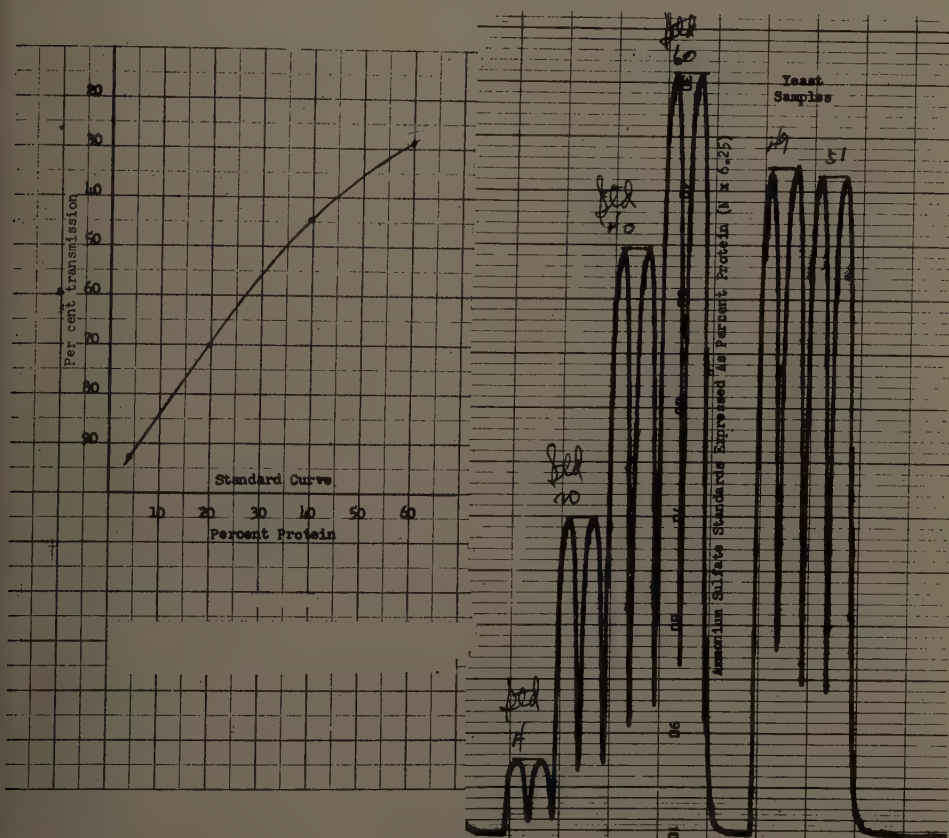


FIGURE 2. AutoAnalyzer plot of ammonium sulfate standards and two yeast samples. The graph at the left is the standard curve for ammonium sulfate solutions.

picked up, segmented with air bubbles (1.2 ml./min.), and sent through a mixing coil. This stream then is mixed with 0.2 per cent 1-amino-2-naphthol-4-sulfonic acid, after which it is passed through a 95° C. heating bath that contains sufficient coiled tubing to cause a delay of approximately 2 min. for color development. The colored product is measured at 641  $m\mu$  in a flow cuvette with a 4-mm. light path.

Typical results are shown in FIGURE 4. Samples 17, 19, and 21 are typical yeast samples; sample 35 is molasses. Standard solutions of potassium phosphate are employed that will give recorder plots for calculated values of 0.3

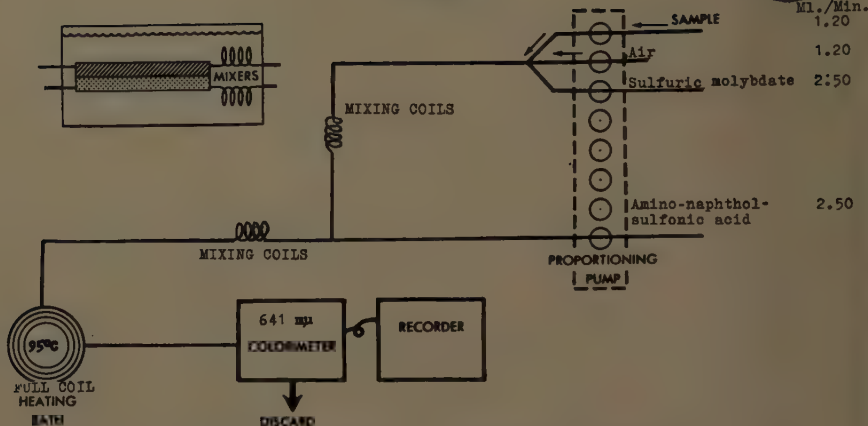


FIGURE 3. Flow diagram for phosphate determination.

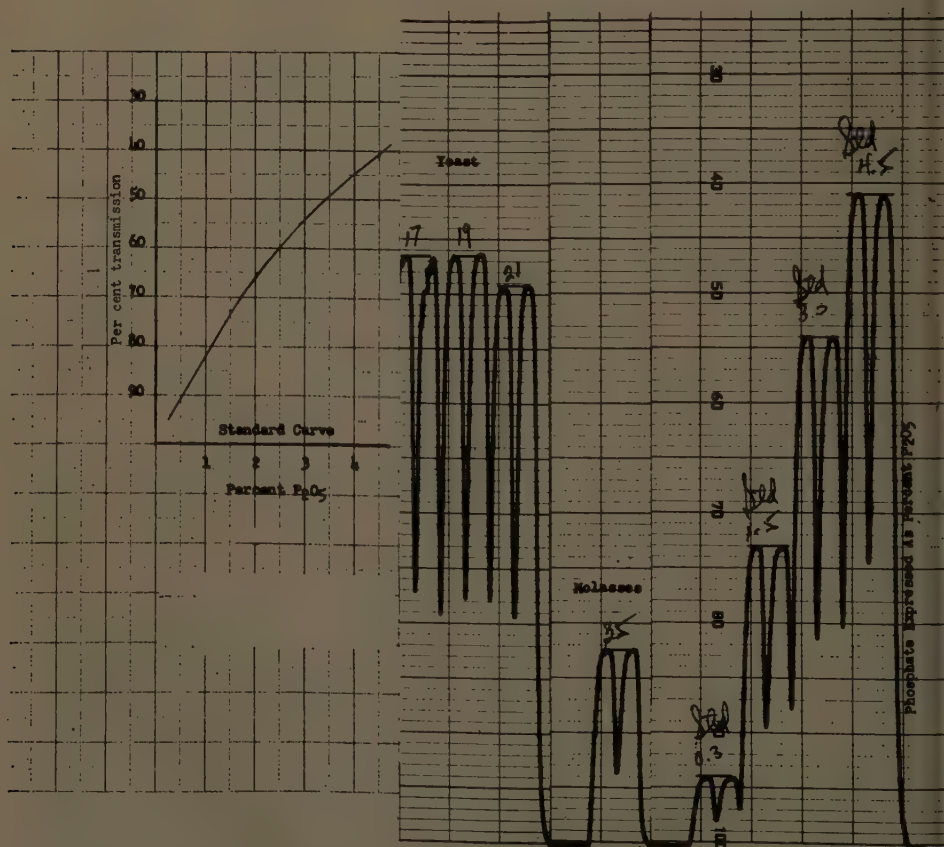


FIGURE 4. AutoAnalyzer data from phosphate determinations of three yeast samples and one molasses sample. A phosphate standard curve is also shown.



to 4.5 per cent  $P_2O_5$ . This range is sufficient for all materials tested in our laboratories. Results are calculated as  $P_2O_5$  values, since this is the unit of measurement that has become classic in the production of bakers' yeast.

### Analysis of Molasses

Analysis of molasses generally is carried out on the clarified and sterilized feeding mash which consists, usually, of a mixture of cane molasses and beet molasses.

*Protein and  $P_2O_5$ .* The feeding mash is analyzed for protein and  $P_2O_5$  by concentrating a measured volume of the mash and following the same procedure employed for yeast analysis.

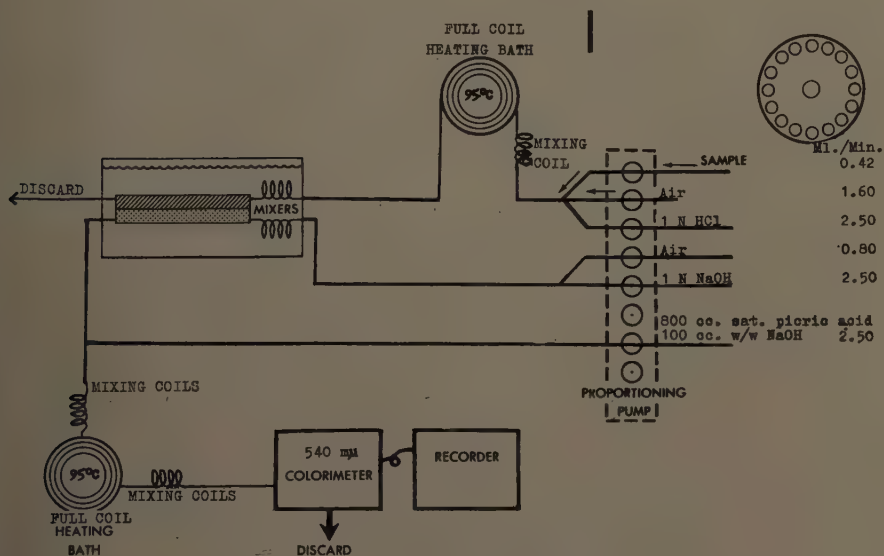


FIGURE 5. Flow diagram for sugar determination.

*Sugars.* The procedure for the determination of feeding mash sugar is shown in FIGURE 5. The sample (0.42 ml./min.) is mixed with 1.0 N HCl (2.50 ml./min.) and air bubbles (1.6 ml./min.). The stream then flows for approximately 3 min. through a water bath held at 95° C. while the sucrose present in the molasses mash is hydrolyzed. The sample then flows through cooling coils to a plate dialyzer at 37° C, where it is dialyzed against a stream of 1 N NaOH (2.50 ml./min.) which also is segmented with air bubbles (0.8 ml./min.). The dialysate stream then is mixed with an alkaline picrate solution flowing at 2.5 ml./min. The mixture passes through a 95° C. water bath for approximately 2 min., and the colored product that develops is measured at 540  $\mu$  in a flow cuvette with an 8-mm. light path. The same procedure has been employed for determination of sugar in grain worts used by the brewery. Data not presented here show that the AutoAnalyzer results agree closely with those from the copper sulfate-reduction method commonly employed.

FIGURE 6 shows typical results obtained when mash samples, reference standards, and water blanks are presented to the AutoAnalyzer. Samples 63 and 64 are molasses feeding mashes carried to a standard dilution.

We have found it best to run separate standards for sucrose and molasses, since results with different samples of molasses have varied. If agreement is not good between the sucrose standards and the molasses samples, the sugar

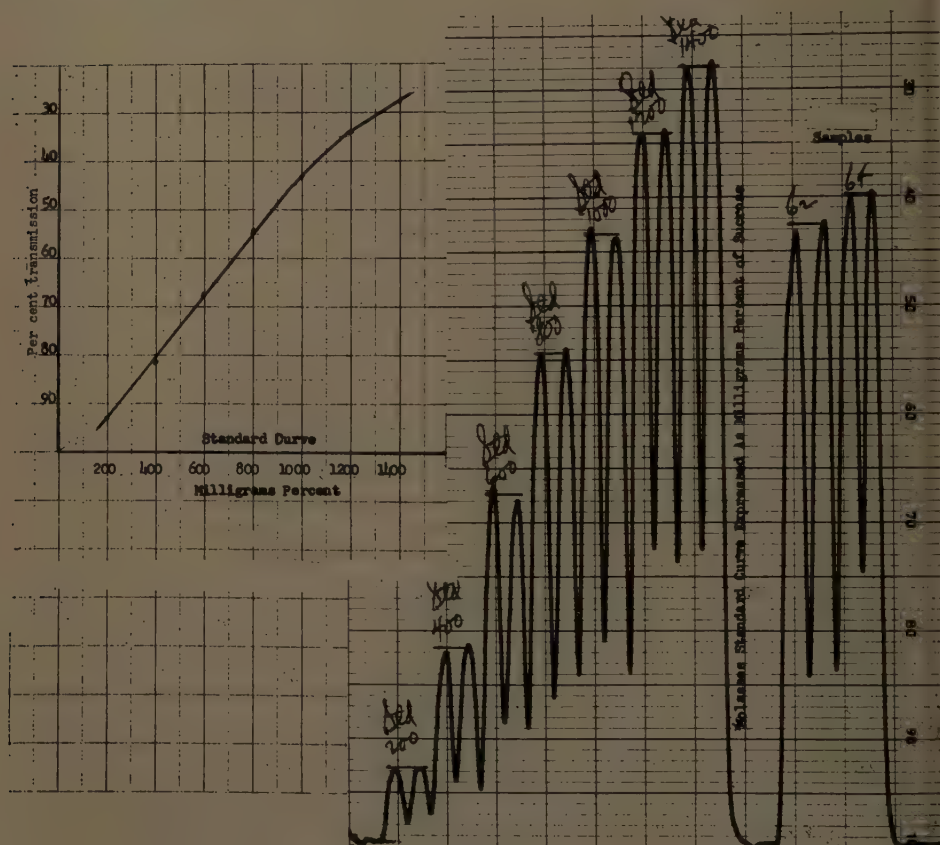


FIGURE 6. AutoAnalyzer plot of molasses standard solutions; plots for two diluted feeding mashes.

content of the molasses is determined by other means. Standard dilutions of the molasses then can be used as the reference standards, replacing sucrose.

#### DISCUSSION

The preliminary digestion of yeast and molasses samples has been found necessary to ensure a high degree of analytical accuracy in the determination of protein. Attempts were made to employ a biuret-reagent colorimetric test of a dialysate from alkali-hydrolyzed yeast. However, excessive variation

was observed in the extent of hydrolysis with phosphate content of the yeast, so the classic digestion technique was adopted. This technique requires simple equipment, a minimum of attention during digestion, and it provides digested material for both the protein nitrogen and phosphate determinations. Use of the AutoAnalyzer to determine ammonia nitrogen saves considerable time and effort, since individual analyses ordinarily take the major part of a technician's time.

Troubles encountered in carrying out these analyses have been few and relatively simple. Most of them are eliminated when reagent-grade chemicals are used and reagent solutions are prepared afresh and kept clear.

#### ACKNOWLEDGMENTS

We are grateful to Andres Ferrari of Technicon Instruments Corporation for working out basic analytical procedures and for offering valuable suggestions for their modification to suit our specific requirements.

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# AUTOMATIC ENZYMATIC ANALYSIS FOR L-LYSINE VIA DECARBOXYLATION

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The large-scale production of amino acids requires that rigid process controls be incorporated at all stages of fermentation and product recovery, not only to establish process performance but also to ensure quality of product. The accomplishment of such control necessitates the analysis of a large number of samples throughout each production cycle. In order to ensure economy of operation, it is necessary to establish monitoring methods superior to conventional chemical and microbiological analyses, which are time-consuming and therefore costly. Since only a limited number of assays can be carried out in one day by the conventional Warburg gasometric method<sup>1</sup> of analyzing L-lysine in fermentation broth, an effort was made to develop an automated colorimetric assay, employing the AutoAnalyzer.\* The resulting assay requires a minimum of human supervision and is rapid, highly accurate, dependable, and reproducible.

The AutoAnalyzer determination of L-lysine in fermentation broth samples is achieved by a continuous colorimetric analysis of the carbon dioxide liberated on a mole-per-mole basis by the enzymatic decarboxylation of L-lysine.

## *General Description*

The flow diagram for the L-lysine determination is shown in FIGURE 1. The initial component, not shown in the flow diagram, is a rotating sampler plate capable of holding forty samples. The broth samples are presented sequentially to a sampling tube that dips in and out of the sample cups at a pre-determined rate.

The automated system is controlled by a multichannel proportioning pump that delivers fixed volume ratios of sample, diluent, enzyme, and reagents to other components in the system. The flow rates in milliliters per minute for each reagent tube are given in FIGURE 1.

A fixed volume of broth is aspirated, diluted with water, segmented with CO<sub>2</sub>-free air and, finally, mixed with an appropriate volume of the enzyme L-lysine decarboxylase. The overflow sampler, situated upstream of the enzyme-substrate mixing, provides small aliquots of the diluted sample. The enzyme and substrate are mixed by repeated inversion on passing through glass helices in the mixer. The stream then passes through time-delay mixing coils in a 37° C. water bath; during passage, the released carbon dioxide enters the air phase. Emerging from the coils, the stream runs into a liquid-gas separator: the liquid goes to waste and the gas phase, which contains carbon dioxide, is aspirated. The gaseous stream now segments a stream of weak alkaline buffer

\* Technicon Instruments Corp., Chauncey, N. Y.



reagent, sodium carbonate and bicarbonate with phenolphthalein. The gas is absorbed by the alkaline solution, and the resulting decrease in  $pH$  is reflected in a reduction in the indicator color. The indicator stream passes along a 6-mm. light path where the optical density (color intensity) is measured in a dual-beam colorimeter at  $555 m\mu$ , the color intensity being inversely proportional to the quantity of L-lysine present in the sample.

### Materials and Methods

**Rate of assay.** The recommended rate of assay is 40 determinations per hour with a wash between each sample. Distilled water is the wash required for assays of L-lysine in complex organic media.

**Diluent.** The diluent consists of distilled water. However, in order to avoid the accumulation of an organic deposit in the polyethylene tubing, it is necessary to add a small amount of a surface-active agent; in this case, Tween 20 was used.

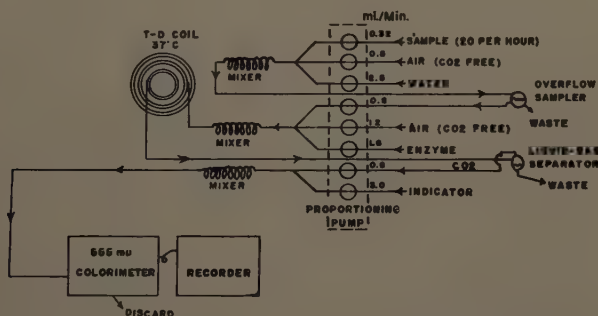


FIGURE 1. Schematic flow diagram for the AutoAnalyzer determination of L-lysine via decarboxylation.

**Enzyme preparation.** The enzyme, L-lysine decarboxylase, is available commercially as the acetone-dry powder.\* The active material is prepared by adding 5 mg./ml. of enzyme to 0.2 *N* phosphate buffer at  $pH$  6.0 and stirring until emulsion is complete. A small amount of antifoam (Dow Corning Antifoam B) is added to the enzyme preparation in order to avoid the formation of bubbles in the liquid-gas separator. For best results a fresh enzyme solution is prepared each day.

**Buffer system.** The buffer system consists of sodium carbonate and bicarbonate, with phenolphthalein as the indicator. The sensitivity of the method can be adjusted to any desired range; for example, decreasing the amount of carbonate-bicarbonate buffer will greatly increase sensitivity (and simultaneously limit, to some extent, the assay range). The procedure for the determination is essentially an extension of the standard AutoAnalyzer determination for carbon dioxide.<sup>2</sup>

**Preparation of standard curve.** In our laboratory the best results have been achieved with standards prepared in fresh, uninoculated lysine fermentation

\* Nutritional Biochemical Co., Cleveland 28, Ohio.

broth rather than in distilled water. Further characterization of this observation has not been carried out. A typical L-lysine standard curve is linear when plotted on semilog paper, as shown in FIGURE 2. It has been found that the standard curve is reproducible from day to day within an over-all error of less than 1 per cent.

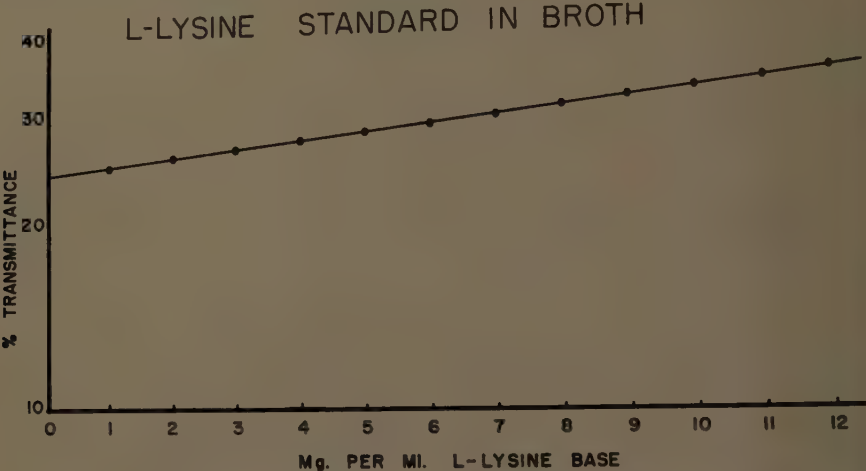


FIGURE 2. Semilog plot of standard solutions prepared in fresh uninoculated L-lysine fermentation broth.

TABLE 1  
ANALYSIS OF A SAMPLE OF L-LYSINE IN FERMENTATION BROTH

Warburg gasometric (mg./ml.)	AutoAnalyzer colorimetric (mg./ml.)
10.48	10.95
10.78	10.96
10.38	10.97
10.37	10.97
10.73	10.95
10.54 avg.	10.96 avg.

TABLE 2  
AUTOANALYZER ANALYSIS OF L-LYSINE IN FERMENTATION BROTH SAMPLES

Sample	L-lysine (mg./ml.)
1	1.3
2	15.21
1	1.4
2	15.4
3	10.65
1	1.37
3	10.91
3	10.80

### *Results*

TABLE 1 presents a comparison of a Warburg gasometric assay and on AutoAnalyzer colorimetric assay for L-lysine. The close agreement between the results of the two procedures is apparent, and the reproducibility of sample analysis is demonstrated. The times required to conduct each of these assay procedures are significantly different, the AutoAnalyzer being considerably more rapid and requiring less attention by the technician.

The experimental data presented in TABLE 2 represent the repeated assay of several samples of broth containing different concentrations of L-lysine. These data illustrate the rather wide range of L-lysine concentrations that may be assayed for via the AutoAnalyzer without the need for dilutions or estimations of broth potency. The reproducibility of assay values when high- and low-concentration measurements are interspersed is also demonstrated in TABLE 2.

### *Summary*

An L-lysine assay has been developed utilizing the AutoAnalyzer. The assay is accurate and dependable and requires a minimum of human supervision. It is based on the colorimetric analysis of carbon dioxide liberated on a mole-per-mole basis by enzymatic decarboxylation of the amino acid.

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# AUTOMATION OF SUGAR ANALYSIS IN BAROMETRIC CONDENSERS AND BOILER WATERS

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Recently an instrument was made available that consists of components designed to measure continuously and compare on a moving graph the concentration of a given substance in the test solution against a known concentration of that substance in a standard control solution. No volumetric or gravimetric measurement is involved, since only ratios are plotted. Most important of all, the operation is completely automatic. This instrument is the Auto-Analyzer.\*

We first developed a method for continuous automatic determination of sugar in steam condensate returning to the power plant.† In our refinery, steam condensed in vacuum pans, evaporators, and various heaters forms a vital source of water supply for the boilers. Sugar may enter these waters through defects in heating surfaces,<sup>1</sup> and the resulting contamination can cause decomposition of the sugars by heat into acids that are detrimental to the steel of the boilers. It is evident that rigorous control of the feed water is the best safeguard against sugar contamination.

This automatic method entails the analysis of low sugar concentrations in pure waters. Although there are serious considerations specific to this application, it is of greater interest to review a method for determining sugars in impure solution.

The method to which I refer is applied to the detection of sugar losses to condenser waters. Certainly, the most important problem confronting the sugar refiner is the question of raw sugar input as compared to market yield. Process losses are a major expense in most refineries. The amount of loss can vary anywhere from 0.33 to 1 per cent of the sugar melted.<sup>2</sup> Losses in excess of 1 per cent have been encountered.

Losses can be classified into two general types: determined and undetermined.<sup>3</sup> Mechanical losses fall into the undetermined category. Entrainment is a mechanical loss, the sugar being carried over from vacuum pans and evaporators to condenser waters in the vapor. The detection of losses of this nature in the refinery always has presented problems of great complexity, owing in particular to the intermittent nature of entrainment.<sup>4</sup> A small percentage of sugar in the condenser waters from the pans and evaporators can result in large monetary losses. For this reason extensive study has been devoted toward detection of sugars in condenser waters.<sup>4-7</sup>

At Refined Syrups & Sugars, Inc., evaporator vapors are condensed by river water in barometric condensers. River water, as an impure solution containing numerous salts and sedimentation, presents certain physical and chemical considerations to be dealt with when the AutoAnalyzer is utilized as a monitoring device.

\* Technicon Instruments Corp., Chauncey, N. Y.

† The adaptation of the AutoAnalyzer for automatic sugar determination in steam condensate was carried out by R. Muraika and E. D. Gillette of Refined Syrups & Sugars, Inc.



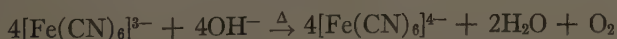
The instrumentation system provides for the complete automation of the steps necessary for analytical sampling, filtration, heating, color measurement, and recording. The system is controlled by a multichannel proportioning pump that advances fixed volume ratios of sample and reagents to other components of the system. Filtration is accomplished by a continuous concurrent dialysis unit, and optical densities are measured by a dual-beam colorimeter with a continuous-flow cell. Detailed descriptions of the instrumentation have been published.<sup>8-10</sup>

Of the many available color reactions of sugars, the alpha-naphthol reaction is employed in the test most widely used for determining the presence of sugar in condenser waters, boiler feed waters, factory sewer outflows, and other waters where the presence of sugar may be undesirable.<sup>11</sup> The sugar solution (1 to 2 ml.) is treated in a test tube with 1 to 2 drops of a 10 to 20 per cent alcoholic solution of alpha-naphthol. A few milliliters of nitric acid-free, concentrated sulfuric acid then are poured carefully down the walls of the tube. When sucrose is present, a violet zone or ring appears at the juncture of the two liquids, the intensity of the color depending on the concentration of sucrose.<sup>12</sup>

To determine sucrose quantitatively by chemical methods it is first necessary to change it into invert sugar by hydrolysis with acids or invertase. If reducing sugars are already present, these must be determined before inversion of the sucrose and deducted from the total found after inversion, the sucrose being calculated from this difference.

The classic Lane and Eynon volumetric method<sup>13</sup> is the most widely accepted laboratory procedure for determining sugars; it is based on the property shared by all aldehydes and ketones of reducing alkaline solutions of certain metallic salts. The method entails the use of the Soxhlet modification of Fehling's solution,<sup>14</sup> which consists of a copper sulfate solution and an alkaline tartrate solution mixed in equal volumes just before the analysis. Methylene blue is used as an internal indicator of the end point. After the invert completely reduces all the copper in a measured volume of Fehling's solution, the methylene blue is completely decolorized by minute amounts of reducing sugar. The reduction is carried out in a flask in which the liquid is kept boiling to prevent reoxidation.

We chose a modification of the method for determining glucose as proposed by W. S. Hoffmann<sup>15</sup> for adaptation to the automatic system. The sugars are inverted totally and determined by a direct reading procedure that utilizes the reduction of the yellow solution of potassium ferricyanide to the colorless ferrocyanide in alkaline solution. Four molecules of ferricyanide furnish two atoms of oxygen, according to the equation



The color is measured at 420  $\mu$  in a flow cuvette with a 6-mm. light path.

The mechanism of this reaction was studied, and valuable results were obtained by Wood<sup>16</sup> on the basis of oxidation-reduction potentials. Some of his findings and how we utilized them are now presented:

(1) By lowering the temperature a retardation of the reaction is effected and

the final amount of oxidant is increased. We therefore operate our heating bath at a temperature of 96° C. to attain a rapid reaction rate.

(2) Lowering the alkalinity brings about the same quantitative result as lowering the temperature. We add NaOH in excess of the neutralization point prior to reduction of the ferricyanide. HCl (0.25 *N* at 0.6 ml./min.) is added for hydrolysis and 0.6 ml./min. of 0.3 *N* NaOH is added before reduction. The *pH* for inversion is 1.5 and for reduction 10.5.

(3) The reducing effect of glucose is increased greatly by the presence of KCN. Since it was found that KCN more than doubles the sensitivity of this reaction, we have eliminated the use of this reagent for the condenser application. Extreme sensitivity is not essential at the sacrifice of a part of the detection range. Normally, 25 ppm is clearly visible with our technique; however, sacrificing some sensitivity we can now observe the range of 0 to 400 ppm. In cases where more sensitivity is demanded, a solution of KCN can be introduced to good effect after dialysis and before reduction.

(4) An increase in salt content also can retard the reaction. We determined experimentally the concentration of salt that must be added to the ferricyanide reagent to compensate for the salt content of the river water, so that the osmotic pressure in the dialyzer would be favorable, permitting diffusion of invert molecules. We found that a 2 per cent NaCl solution was sufficient for satisfactory diffusion.

It is of value also to note the effect of temperature on dialysis as seen in FIGURE 1. For every degree rise in temperature there is a 3.5 mg. per cent increase in invert diffusion. This proportionality is valid until the equilibrium point of diffusion is approached, at which 50 per cent of the total invert has diffused from the sample to the recipient stream. Although this diffusion-versus-temperature relationship is expressed at levels of mg. per cent, the corresponding percentage would be the same at lower levels of concentration. They would correspond to 35.0  $\mu\text{g./ml.}$  We operate our dialyzer at a temperature of 65° C. and achieve excellent sensitivity.

Another consideration to be dealt with concerning dialysis is the effect of area on diffusion rate and concentration of the diffusate in the recipient stream. This is illustrated in FIGURES 2 and 3, for a single dialyzer and one of twice the area, respectively. It is evident from these curves that the use of an additional dialyzer provides an excellent means of attaining additional sensitivity. Experience has shown, however, that for levels of 2.5 mg. per cent sugar in the sample stream, one dialyzer at 65° C. provides adequate diffusion and sensitivity.

In analyzing a sample of our river water we detected a particularly high magnesium content that necessitated the establishment of acidic conditions in the dialysis stage to prevent precipitation of magnesium hydroxide in the dialyzer that would interfere with flow and diffusion. Since the reduction of ferricyanide by invert sugar requires basic conditions, it was necessary to introduce a separate stream of NaOH after dialysis and before heating. In the event that there are no constituents in the sample stream that will precipitate at a high *pH*, a basic ferricyanide reagent can be used, obviating the need for an additional stream.

We were troubled also with varying amounts of dissolved iron in the condenser water. Prussian blue formed in the dialyzer as a result of the chemical reaction of dissolved ferrous iron with the ferricyanide reagent at low  $pH$ , and prevented free diffusion of invert sugar across the pores of the dialyzer membrane. This situation was corrected by the addition of a small amount of sequestering agent, Versene Fe-3, obtainable in both powder and liquid state

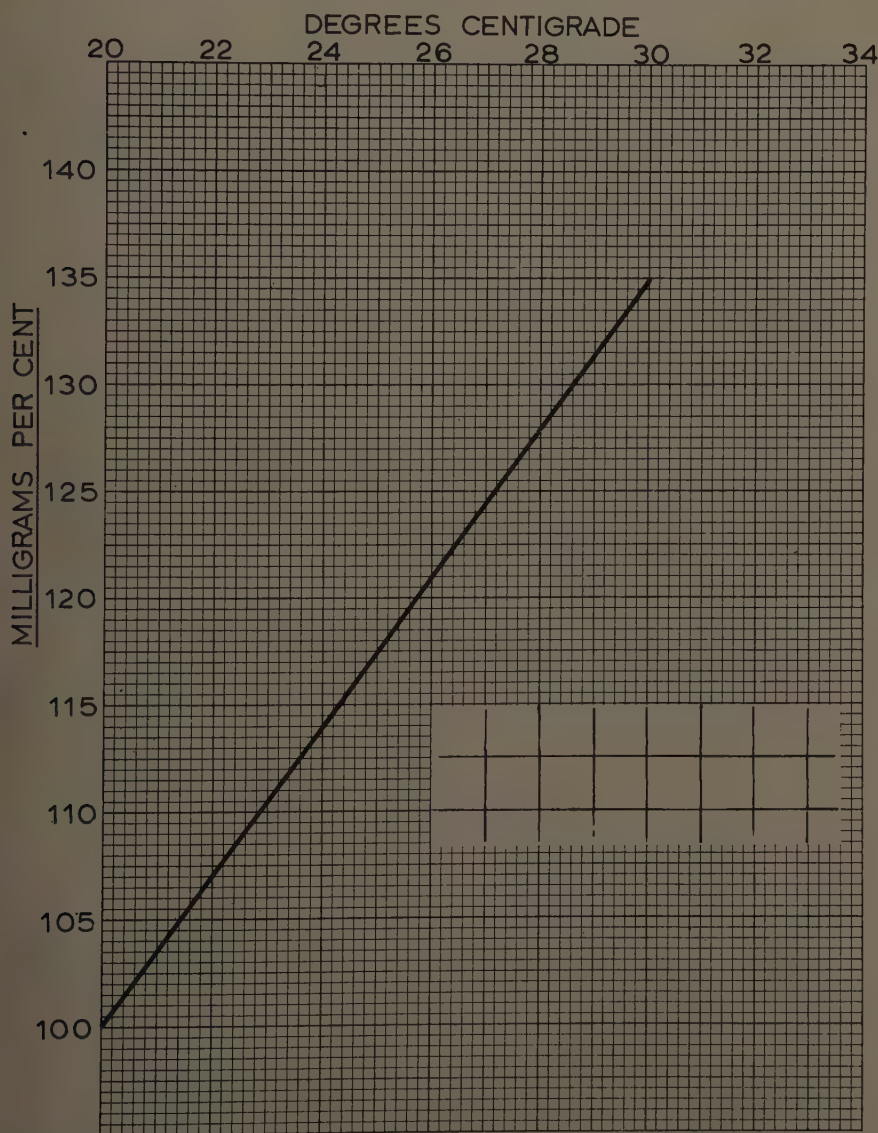


FIGURE 1. Glucose dialyzed versus temperature at 100 mg. per cent level. Slope: 3.5 mg./°C.

from the Dow Chemical Company. The Fe-3 combines chemically with ferric or ferrous ions to form a product that is completely stable and soluble in the acid-to-alkaline pH region.

The river water picked up by the pumps and circulated through the condensers contains varying amounts of sugar, depending on tide conditions. Sugar contamination of the river is due to various sources in the refinery. This

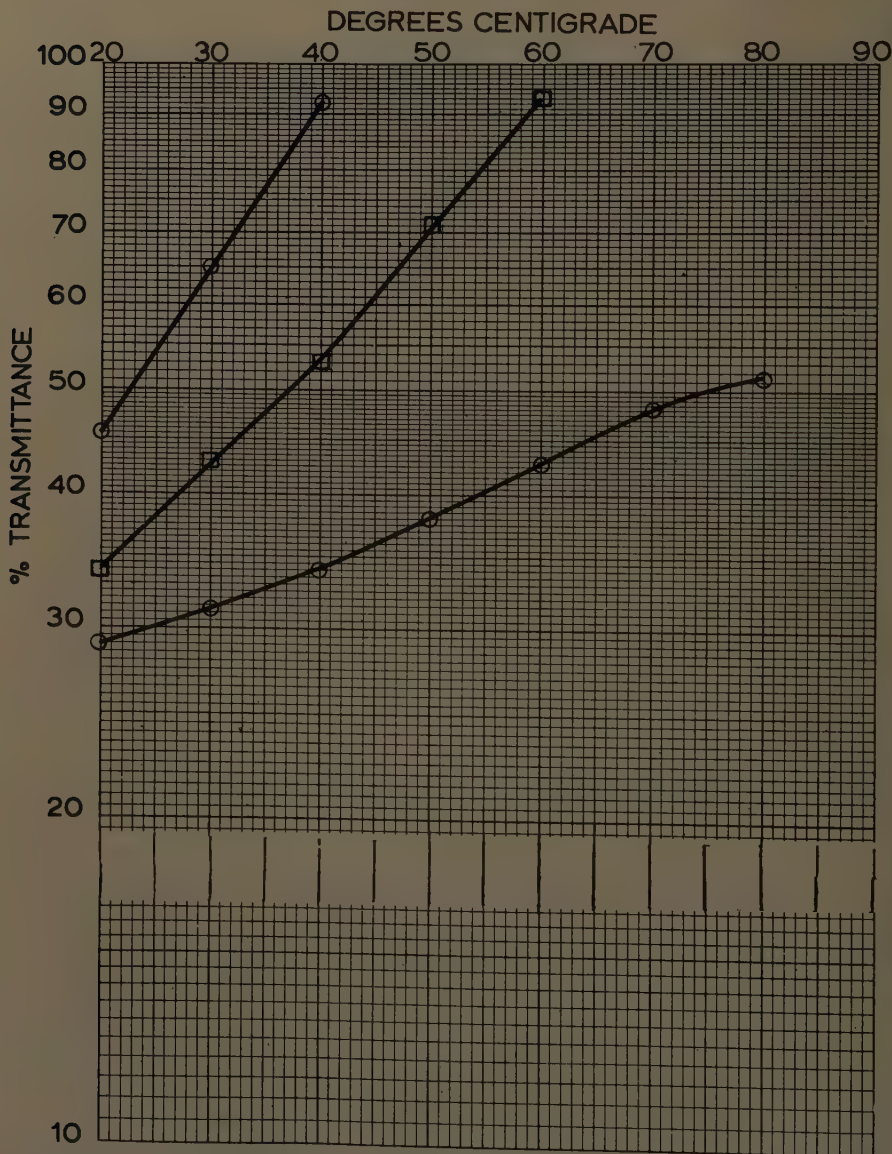


FIGURE 2. Transmittance of glucose dialyzed in one dialyzer against (top to bottom) 50 mg. per cent standard, ○, 100 mg. per cent standards □, and 150 mg. per cent standard, ○.



situation necessitated the use of two analyzers, one at the condenser, the other at the river-water inlet. The instruments operate identically. The absolute sugar content in condenser water is calculated by subtracting the optical density of the incoming river water from the outgoing condenser water and relating the difference to a standard graph.

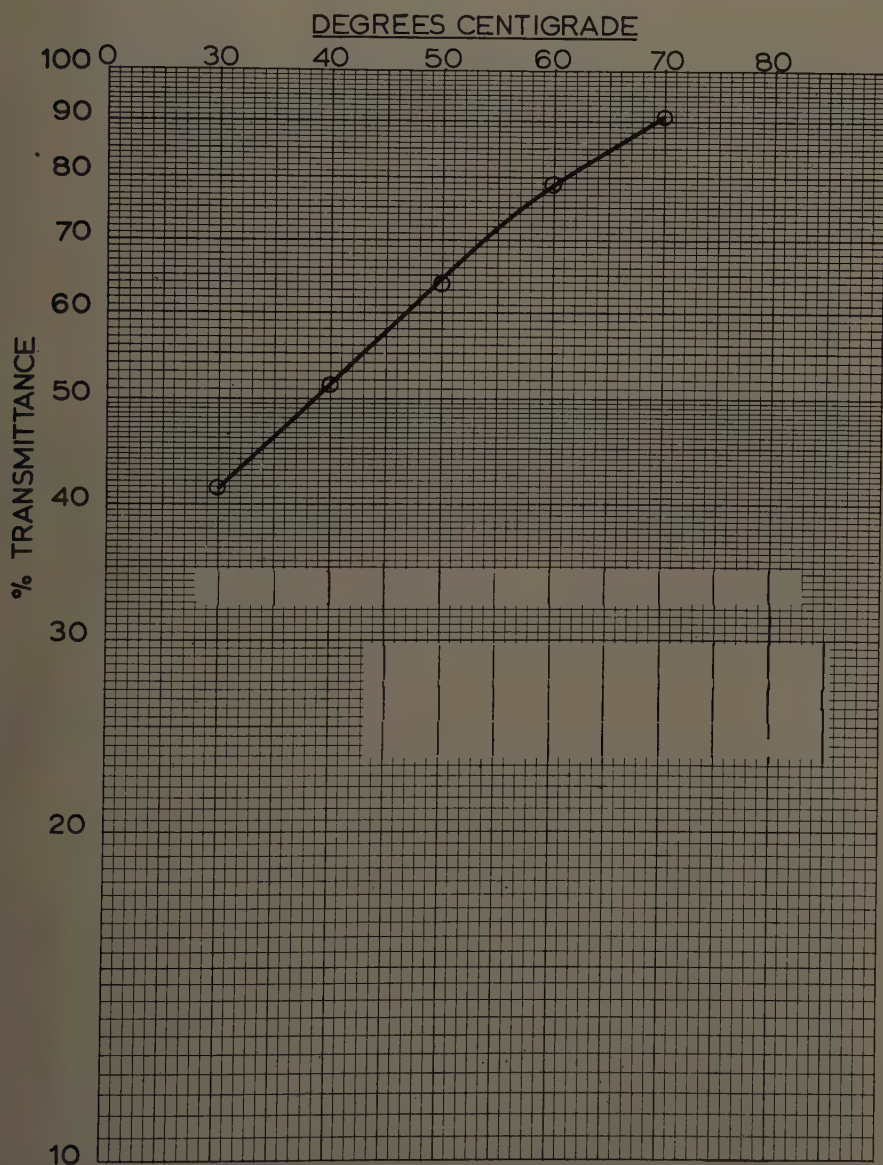


FIGURE 3. Transmittance of glucose dialyzed in double dialyzer against 50 mg. per cent glucose standard.

The procedure as performed by this system for the determination of total sugars starts with the automatic proportioning of reagents and sample as seen in FIGURE 4. Condenser water (2.0 ml./min.) is joined by 0.6 ml./min. of 0.25 *N* HCl and segmented by 2.5 ml./min. of air. The acidic solution enters a heating bath at 96° C. for hydrolysis of any sugars present. The flowing stream of condenser water and invert enters the upper half of the dialyzer at 65° C. Two milliliters per minute of 0.075 per cent  $K_3Fe(CN)_6$  in 2 per cent NaCl solution is segmented by 1.2 ml./min. of air and enters the lower half. In the dialyzer the invert molecules diffuse through a semipermeable membrane (D30 type) to enter into the ferricyanide stream. At the end of the circuit the re-

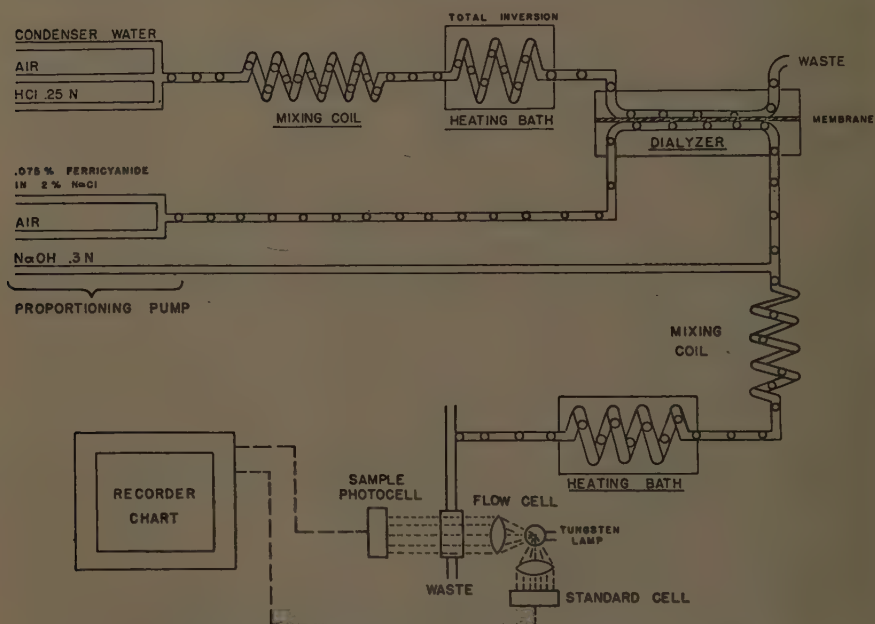


FIGURE 4. Schematic of system for determining total sugar in condenser water.

gent stream of  $K_3Fe(CN)_6$  emerges containing a portion of the total concentration of the sugar in the condenser water. The remainder of the sample passes off to waste. In this manner the dialyzer serves to filter out any sedimentation. Time and area of dialysis exposure being constant, the total amount of sugar diffused varies only as its concentration. A stream of 0.6 ml./min. 0.3 *N* NaOH mixes with the diffusate to raise the *pH* for reduction.

The basic solution of ferricyanide and diffused invert then enters the heating bath at 96° C., where reduction of yellow ferricyanide to colorless ferrocyanide occurs. This reduction is proportional to the amount of invert present in solution.

The stream issuing from the heating bath enters the colorimeter through a flow cuvette with a 6-mm. light path. As the concentration of sugar varies, the colorimeter optically measures changes in transmission at 420  $m\mu$ . The

recorder automatically graphs the results on a moving chart. The time lapse between sampling and recording is normally 10 min.

Standardization with known concentrations of sugar should be performed once a day to ensure accurate quantitative interpretation. A standard curve for aqueous sugar standards is not reproducible from day to day since, as the

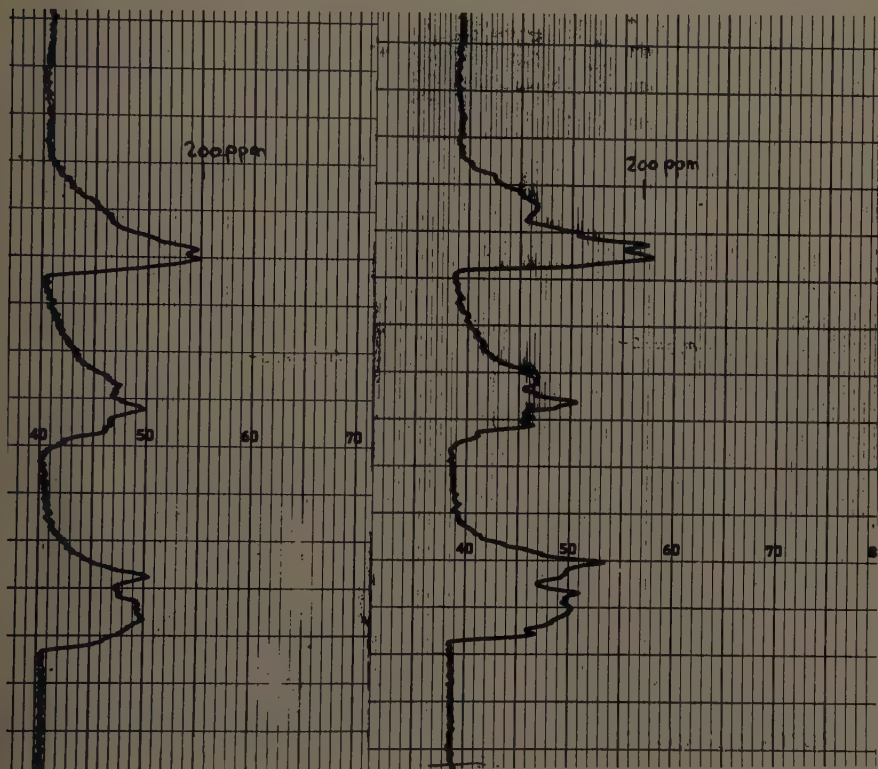


FIGURE 5. Record of sugar present simultaneously in incoming river water (*left*) and condenser water (*right*).

dialyzer membrane and manifold age, there is a corresponding decrease in diffusion and flow rates and a resultant decrease in sensitivity.

### Discussion

Essentially, the advantage of the AutoAnalyzer is its ability to observe and record levels of sugar content continuously. In this respect, it has met our demands. An independent evaluation of the AutoAnalyzer system must be made by each refiner, since different plant conditions will require alterations in the chemistry and mechanics of the method to satisfy individual needs. The value of any conclusions depends fundamentally on the amount of reliable data accumulated. Our conclusions must be tentative, since our investigations

have not yet covered all possible sources of loss. To date, we have encountered limited evidence of losses to condenser waters.

A typical set of charts for comparing the sugar contents of incoming river water and condenser water over the same period of time is seen in FIGURE 5. The curves are almost identical, which indicates efficient operation of the evaporator. Note, however, that the deflections on the condenser graph are greater. This is due to a slightly higher degree of sensitivity of this instrument. The extent of this difference in sensitivity is predetermined by standardization. A

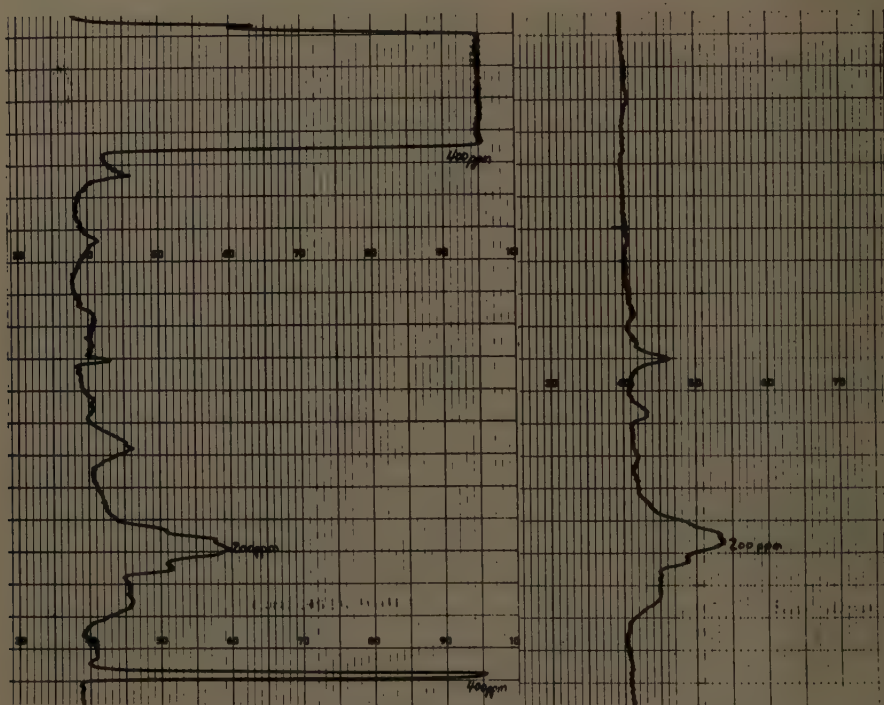


FIGURE 6. Record of sugar present simultaneously in condenser water (*left*) and incoming river water (*right*). Note sugar losses to condenser water.

second set of charts can be seen in FIGURE 6. Losses to condenser water are recorded on two occasions.

#### *Acknowledgments*

My thanks are due to E. D. Gillette, Manager of the Process Development Department, Refined Syrups & Sugars, Inc., for his helpful advice and many valuable suggestions, and to A. Ferrari, Director of the Research Laboratories, Technicon Instruments Corp. for providing invaluable statistical data as well as the illustrations on dialysis that originated in a study in preparation at said laboratories.



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# METHODS DEVELOPMENT FOR PHOSPHATE ANALYSIS WITH THE AUTOANALYZER

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## *Introduction*

Although the AutoAnalyzer\* has been employed for the analysis of phosphate at the parts-per-million level in boiler and condensate waters,<sup>2</sup> a different experimental approach is required for the analysis of phosphate systems in detergent products. In this paper are discussed important aspects of the AutoAnalyzer that required consideration in the development of analytical methods for these phosphate mixtures. The methods are the selective determination of orthophosphate,<sup>6</sup> the determination of total inorganic phosphate,<sup>6</sup> and the application of the AutoAnalyzer to automated ion-exchange chromatography of phosphate mixtures.

Subsequent investigations in this laboratory with flow schemes involving periodate oxidations, diazotizations, enzymatic degradations, turbidimetric processes, and iodometric reactions have been simplified considerably by observance of the principles discussed. These principles can be presented in logical order in discussions of the dialyzer module, the colorimetric system, and the introduction of a specific separation module, an ion-exchange column.

## *Instrumentation*

*Dialyzer module.* Flow schemes that do not utilize the dialyzer module are not applicable to the analysis of phosphates in detergents. Attempts to process heavy-duty commercial detergent products without the dialyzer generally result in voluminous sudsing and turbidity in the flow cell. Although the efficiency of the nonequilibrium dialysis process is only about 10 per cent in the phosphate methods, ample sensitivity and interference-free operation with untreated samples were obtained by consideration of the following principles:

(1) Flow rates on both sides of the cellophane membrane should be no higher than are required for adequate separation of samples. The lowest flow rates that yield identical peak-height responses from consecutive sampling of identical solutions are used in the phosphate methods. Both phosphate methods require the use of two dialyzer plates in series for sufficient sensitivity at these flow rates. Attempts to obtain similar responses with a single dialyzer unit resulted in severe washing problems at the necessarily lower flow rates. Similar washing problems resulted from attempts to provide additional membrane area by use of a single dialyzer with wide-bore passageways.

(2) Flow rates on both sides of the membrane should be significantly different in order to eliminate periodic concentration fluctuations. These irregularities can originate in alternating in-phase and out-of-phase opposition of liquid segments. In both phosphate methods this velocity difference between streams is provided by the use of recipient streams that move faster than the sample streams.

\* Technicon Instruments Corp., Chauncey, N. Y.

(3) It is especially important that the segmentation pattern of liquids and air bubbles be preserved in streams passing through the dialyzer. If this regularity is lost, subsequent addition of reagents at a fixed flow rate results in concentration differences that are often troublesome. Addition of a suitable wetting agent to reagents or diluents is often helpful in obtaining smooth flow; both phosphate methods utilize a wetting agent in the solutions that are pumped into the recipient side of the dialyzer. Routine analysis of phosphates in detergent solutions maintains smooth flow on the sample side of the membrane.

Irregularities in proportioning due to poorly designed flow schemes are not eliminated by diffusion within the dialyzer, but are reflected immediately in the parallel stream on the other side of the membrane. A "last resort" technique for obtaining noiseless recordings is provided by the use of countercurrent flow in the dialyzer. This procedure was examined briefly in the development of the phosphate methods; even under nonequilibrium dialysis conditions this type of flow resulted in a recirculation of phosphate that eliminated severe fluctuations in phosphate concentration, but detectable contamination between samples occurred even when samples were introduced at 15-min. intervals. It was concluded that countercurrent flow is not generally advisable because permissible sampling rates are reduced, and lessened peak-height sensitivity results. For these reasons both phosphate methods employ concurrent flow in the dialyzer to permit high sensitivity at 20-per-hour sampling rates.

(4) It is desirable that a dynamic steady state be maintained in the dialyzer. For this reason the volume delivery of sample solution should be kept small in comparison with the volume delivery of its diluent. Monitoring situations do not involve intermittent addition of sample solutions and, consequently, the sample solution and diluent may be delivered in any convenient volume ratio.

(5) The concentration range over which dialysis is a linear function of concentration often can be broadened by maintaining a high electrolyte concentration in the dialyzer. In the orthophosphate method sufficient ionic strength is provided by a sodium sulfate solution, while sulfuric acid introduced at an earlier stage serves the same purposes in the total phosphate method.

(6) Use of a reagent solution to provide the desired electrolyte concentration during dialysis is permissible, but the effects of possible dilutions and changes in the reagent's composition should be anticipated. For example, an acidic solution of ammonium molybdate has a lower acid:molybdate ratio after passing through a dialyzer with distilled water on the other side of the membrane. Composition changes like these often invalidate colorimetric systems.

(7) Precipitation reactions should never be allowed to occur within the dialyzer passages and should be avoided generally throughout the rest of the AutoAnalyzer system. The possibilities of precipitation generally can be detected by continuous sampling at a very high sample concentration. The recorder response should reach its equilibrium value rapidly and maintain it; any other behavior indicates the necessity for changing the flow scheme. Both phosphate flow schemes use postdialysis molybdate addition to avoid fouling of the dialyzer membrane with insoluble molybdates.

(8) The flow scheme should take advantage of any enrichment of the sought constituent in dialysis. In the selective analysis for orthophosphate, dialysis

increases the concentration of orthophosphate in relation to the concentrations of other species. As a result, the colorimetric determination is conducted in the presence of reduced interferences.

It is not possible to employ the same principle in a total phosphate flow scheme because enrichment in dialysis could result in a dialyzed sample with a distribution of phosphate species different than the original. As a consequence, it is necessary to convert condensed phosphates to orthophosphate before dialysis.

(9) In certain situations it is possible to use dialysis for addition or elimination of a reagent as well as for the usual purposes. A desirable increase in sensitivity resulting from minimum dilution of the sample is obtained in these cases. This technique was used in the total phosphate flow scheme; most of the sulfuric acid present at high concentration (previously introduced for the automated acidic degradation of condensed phosphates) is eliminated by dialysis. The remaining sulfuric acid provides most of the acidity for the subsequent colorimetric process.

(10) The finished flow scheme should be tested to ascertain that the membrane does not require "preloading" with the sought constituent before reproducible results can be obtained. Several experimental flow schemes for orthophosphate did not function correctly until several samples had been processed. This adsorptive capacity of the membrane for a few micrograms of phosphate did not occur when a high electrolyte concentration was maintained during dialysis.

*Colorimetric system.* The choice of a colorimetric system for an AutoAnalyzer flow scheme is often limited by the nature of the samples to be analyzed. For example, concentrated volumetric solutions of detergent products are troublesome to prepare and difficult to sample and, as a consequence, the original sample solution is restricted to product concentrations of 1 per cent or less. Only limited volumes of these solutions can be introduced into the instrument, and tenfold "dilutions" occur in dialysis. It is evident that the additional dilutions by reagent additions will result in phosphate concentrations in the flow cell of only several parts per million. It was necessary to select very sensitive colorimetric systems for measuring these concentrations in a cell with a path length of 1 cm. or less. Molybdenum blue reactions<sup>1,3</sup> provided adequate sensitivity.

It appears that almost any aqueous colorimetric system can be adapted to the AutoAnalyzer, but several important factors must be considered:

(1) Heating of some colorimetric systems to obtain additional sensitivity is obviously inappropriate. Rapid depolymerization of condensed phosphates in hot acid solutions prevents any use of heat in a selective orthophosphate analysis. In situations where heat is used in an AutoAnalyzer flow scheme, the process should be examined critically to make sure that undesirable side effects have not appeared simultaneously. For example, in one experimental flow scheme it was found that formation of phosphomolybdic acid proceeded smoothly below 40° C., but gave rise to a variety of products at higher temperatures. Subsequent installation of a cooling coil for temperature control at the moment of molybdate addition eliminated this difficulty.



(2) Calibration of the deliveries of the tubes in the proportioning pump is a useful preliminary to rigorous scaling-down of a colorimetric system. However, even when conditions in the continuous-flow system are matched exactly with the classic colorimetry, it is sometimes found that insufficient sensitivity is obtained. The use of longer flow cells might seem attractive in these situations, but the additional volumes of these cells introduce washing problems that make every sensitivity gain harder to obtain. Best results in the phosphate flow schemes were obtained by modifying the colorimetric systems for high sensitivity with 0.8-cm. flow cells.

Extensive changes within the framework of a colorimetric system often provide a great increase in sensitivity, but these changes must be planned systematically with an awareness of the function of every reagent in the system. Minimum dilution of the sample and increases in reagent concentrations provide most of the sensitivity in the phosphate flow schemes, but the ratio of the concentrations of sulfuric acid and ammonium molybdate controls the validity of the measurements. Determination of an optimum value of this ratio was required for these systems; intensely colored reagent blanks, failure to respond to orthophosphate, nonselectivity, and nonlinearity can result in phosphate systems when this ratio is disregarded.

(3) In some colorimetric procedures, the order of addition of reagents can be changed to avoid precipitation. This technique was employed successfully in the selective orthophosphate analysis and appears generally applicable (a) if the reagents do not react with each other, and (b) if the desired consecutive reactions have successively smaller reaction rates.

(4) Chemical equilibria must be considered. For example, since there is no substitute for quantitative acidic degradation of a phosphate mixture of variable composition, this operation must be completed. Considerable processing time can be saved by appropriate use of dynamic steady states when they are applicable. Both phosphate flow schemes involve colorimetric systems with transmission measurements performed before maximum color development has occurred. The measurements are valid because they are conducted at a fixed time interval after the color reaction begins.

(5) Some colorimetric systems present problems that require that the analyst develop specialized techniques and accessories for his flow schemes. Typical colorimetric innovations might include photosensitive processes, detection schemes based on reaction-rate differences, or applications of differential colorimetry. Specialized accessories could include apparatus for controlling gas pressures or for dispersing transient precipitates. An ion-exchange attachment that permits automatic separation of phosphate mixtures will be described as an example.

*Incorporation of a separation module.* Attempts were made to develop additional flow schemes to simplify the analysis of phosphate mixtures. Flow schemes for conducting the selective alkaline hydrolysis of tripolyphosphate<sup>7</sup> were investigated, but continued examination revealed that this approach is probably not applicable to analysis of detergent mixtures because of catalytic decomposition of pyrophosphate.<sup>8</sup>

It was decided then that a separation module could be added to the Auto-

Analyzer system so that each phosphate species would be measured without interference from the others. Fractionations by differences in dialysis rates of condensed phosphates did not appear feasible in view of the low enrichment ratios observed in methods development. Paper chromatographic methods appeared unsuitable for adaptation to the milligram levels required by the total phosphate flow scheme. Ion-exchange chromatography by the methods of Kolloff<sup>5</sup> or Grande and Beukenkamp,<sup>4</sup> with instrumented control of flow rate and elution gradient and instrumented analysis of effluent, offered the most promising possibilities.

Three factors facilitated instrumentation of the separation process:

(1) It was possible to revise the total phosphate flow scheme for greatly increased sensitivity in a monitoring application. This gain in response permitted the use of semimicro-scale separations which could be completed in an hour with conveniently small columns.

(2) Delays of fraction collection were eliminated by pumping the entire column effluent directly into the AutoAnalyzer.

(3) The use of an exponential elution gradient provided elution curves that permitted rapid calculation of a phosphate mixture's composition.

The sensitivity increase was obtained simply by introducing more sample solution into the instrument without changing the dynamic steady states. This was achieved by interchanging the small-diameter sampling tube with the larger-diameter diluent tube.

Similar improvisations for increased sensitivity in monitoring applications are adaptable to most AutoAnalyzer flow schemes if sufficient sample solution, reasonably free from interferences, is available. In ion-exchange chromatography of phosphates the sample solution is available as rapidly as it is pumped from the column, and the background electrolyte is only a dilute potassium chloride solution. Proper operation of the total phosphate flow scheme at the high sample flow rate is ensured by increasing the concentration of the sulfuric acid diluent to compensate for its smaller flow rate.

The resulting combination of a gradient elution system, an ion-exchange column, and a total phosphate flow scheme into one continuous system solved the problem of analyzing phosphate mixtures in detergents. This system comprises two coordinated sections: an AutoAnalyzer for continuous measurement of total phosphate concentration as shown in FIGURE 1, and a separation module, shown in FIGURE 2, for isolating individual phosphates. When the sample turntable of the AutoAnalyzer is replaced by the separation module, the operation of the resulting system is completely automatic after the introduction of a sample.

### *Discussion and Conclusions*

The one inflexible rule for methods development with the AutoAnalyzer system appears to be that all processes must supplement each other. The analyst must consider the complex interrelationships between flow rates, in order to design a flow scheme that satisfies both the requirements for sensitivity and those for fluid dynamics.

Efficient use of combined unit operations eliminates unnecessary dilution of

samples and permits use of colorimetric systems operating at their highest sensitivity. The resulting flow schemes are fast, efficient, and dependable, but often bear little resemblance to their classic equivalents because some reac-



FIGURE 1. AutoAnalyzer arrangement for total phosphate analysis.



FIGURE 2. A typical separation module: an ion-exchange chromatographic system.

tions may not be carried to completion, while others may be conducted uniquely in unfamiliar sequences.

Addition of a separation module such as an ion-exchange chromatographic system permits automatic analysis of multicomponent mixtures. The possibilities in the use of such separation modules are obvious, but considerable ingenuity on the analyst's part may be needed in modifying classical separation processes to supplement AutoAnalyzer detection schemes. It is frequently

possible to modify both the detection scheme and the separation process to achieve the desired results in a continuous-flow system.

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# AUTOMATIC ANALYTICAL INSTRUMENTATION IN THE MODERN POWER PLANT

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## *Introduction*

This paper is neither an account of some outstanding achievement in the field of analytical instrumentation nor a profound discussion of some special aspect of instrumentation theory or practice. It is, instead, a general discussion of the role of automatic analytical instrumentation in the modern electric power plant—the types of instrumentation currently in use, the characteristics of a good instrument system, and the special types of instrumentation now required and being developed for use in new stations, including the Indian Point Nuclear Power Plant.

It is the purpose of this paper to bring to those who are involved in the general field of instrumentation a better understanding of the problems involved in this particular area of application.

## *Background*

Not too many years ago a typical power plant consisted of a collection of boilers, each producing up to several hundred thousand pounds of steam per hour at pressures of 400 to 500 psi. The major portion of the steam produced, after passing through a turbine, was condensed and returned to the boiler. Losses were made up by the addition of softened water to the cycle. The boilers, operating under these conditions of pressure, temperature, and flow rate, had a minimum of "allergies." If fed with condensate and softened water and treated with such materials as sodium hydroxide and disodium phosphate they produced steam of satisfactory purity when the dissolved solids content of the boiler water was kept to less than a few tenths of 1 per cent by blow-down.

Now the picture has changed. The operation of these older boiler plants is no longer economically feasible except, possibly, for backing up modern units during peak loads. How do these modern units compare with their ancestors? The flow diagram, FIGURE 1, shows a typical modern high-pressure unit. The major evolution has been in respect to size, temperature, and pressure. Units generating over 2 million lb. of steam per hour at pressures of more than 1800 psi and temperatures of over 1000° F. are not uncommon today. The efficiencies of these units are in marked contrast to those of their predecessors. However, along with the reduced cost per kilowatt hour of electricity generated have come many operating problems. Let us consider a few of these.

First, with the increase in operating pressures and temperatures and the accompanying increase in heat transfer rates, additional metallurgical problems have developed. Corrosion rates are increased. Relatively thin deposits on heat transfer surfaces can cause thermal stresses, produce concentration potentials, or cause other difficulties that lead eventually to metal failure. Trace concentrations of various materials in the boiler feed water can ultimately lead to serious problems.

Second, the increase in flow rates magnifies the effect of what normally would be considered insignificant phenomena. For example, an oxygen concentration of 0.1 ppm in the condensate of a unit generating 2 million lb. of steam per hour can theoretically remove nearly 20 lb. of iron per day by corrosion and deposit this iron oxide in some adverse location in the system.

Third, the reduced blow-down rates used with these modern units to prevent thermal losses causes a rapid build-up and high-equilibrium concentration in the boiler of materials appearing in the feed water in low concentrations. For example, with a blow-down rate of 0.25 per cent, the concentration would be 400 to 1.

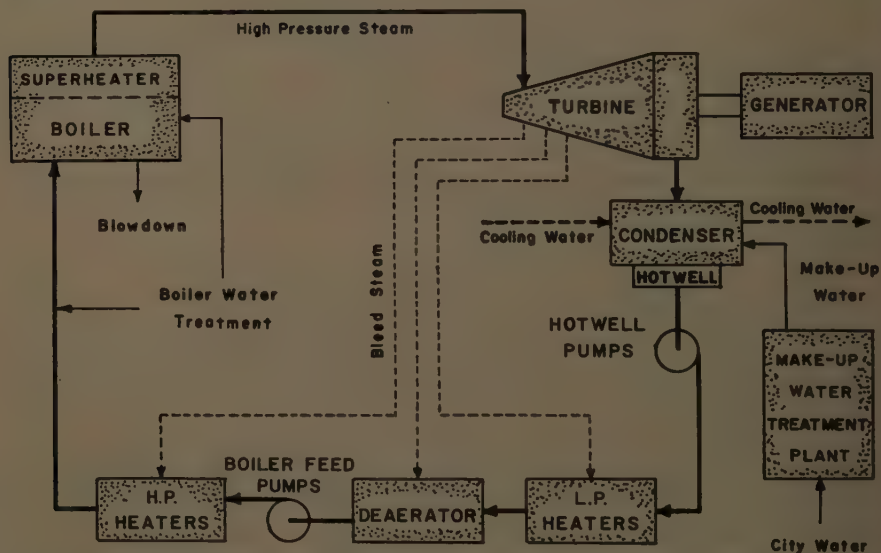


FIGURE 1. Typical high-pressure steam electric generating station. Simplified steam condensate, and make-up water flow diagram.

Finally, the higher temperatures in the boiler drum and the higher evaporation rates increase the difficulties in maintaining steam purity. The vapor pressure of such materials as silica becomes appreciable at these elevated temperatures. This situation and mechanical carry-over or both can cause turbine fouling. Even when the quantity of material is small, a seemingly thin deposit on the blades of a modern turbine can cause an appreciable decrease in efficiency.

The net result of this situation is that water of a purity fantastic by conventional standards must be used for make-up, and trace concentrations of various trouble-making materials must be monitored in the plant streams. When deviations from the normal conditions occur they must be spotted immediately. The source of the difficulty must then be located and corrective action taken.

In the older plants, a few tests per day manually performed on boiler water and other plant streams were adequate. In modern plants, the common practice has been to use improved manual tests at a greater frequency, supplemented by the use of some of the established types of analytical instrumenta-

tion such as conductivity,  $pH$ , and oxygen recorders. In many cases this has proved inadequate. If the present trends in power plant design continue, analytical instrumentation will play an increasingly important role in plant operation.

FIGURE 2 shows the control panel of the water treatment plant in a modern electric station. Among the instruments shown are conductivity and  $pH$  recorders, which are used to guide the operator in controlling plant operations. FIGURE 3 shows part of the sampling system in the same station. A variety



FIGURE 2. Control panel of the water treatment plant in a modern electric station.

of instruments of the manually operated type is available for use in analyzing the various plant streams.

A few examples of the expanding role of analytical instrumentation are discussed below.

#### *Conductivity and $pH$ Measurement*

At one time  $pH$  and conductivity determinations could be made on grab samples with a sufficient degree of accuracy by the use of the simpler types of manually operated test equipment. Today, the extreme purity of the samples involved (frequently having conductances as low as  $0.1 \mu mho/cm.$ ) requires that most determinations be made on flowing samples in closed flow cells to prevent atmospheric contamination. This has led to an increase in both the

number of installed recording instruments and in the use of portable flow-type indicating and recording instruments.

### *Oxygen Analysis*

The determination of dissolved oxygen was usually accomplished by means of manual tests periodically performed. Oxygen recorders were often used in a few key locations. These instruments usually employed the principle of thermal conductivity measurements on hydrogen that had reached equilibrium



FIGURE 3. Analytical instruments used in conjunction with the sampling system of a typical modern electric station.

with the sample. However, the dependence of oxygen concentration upon the operating conditions of various items of plant equipment plus the increase in the importance of that oxygen concentration increases the need for recording oxygen analyzers. Various volatile amines and filming amines are often used to alleviate the effects of oxygen. Hydrazine is used in some plants to reduce chemically the oxygen concentrations. The beneficial aspects of such treatments, whatever they may be, are lessened somewhat by the difficulties that these materials cause in the measurement of the residual dissolved oxygen. Newer types of oxygen analyzers are being developed in an attempt to overcome this problem. An instrument of this type, made by the Cambridge Instrument Company, is shown in FIGURE 4.



*Colorimetric Analysis*

The methods most commonly used for the determination of such materials as silica, iron, or phosphate in low concentrations are based upon colorimetric procedures. The common practice formerly was to take a grab sample and

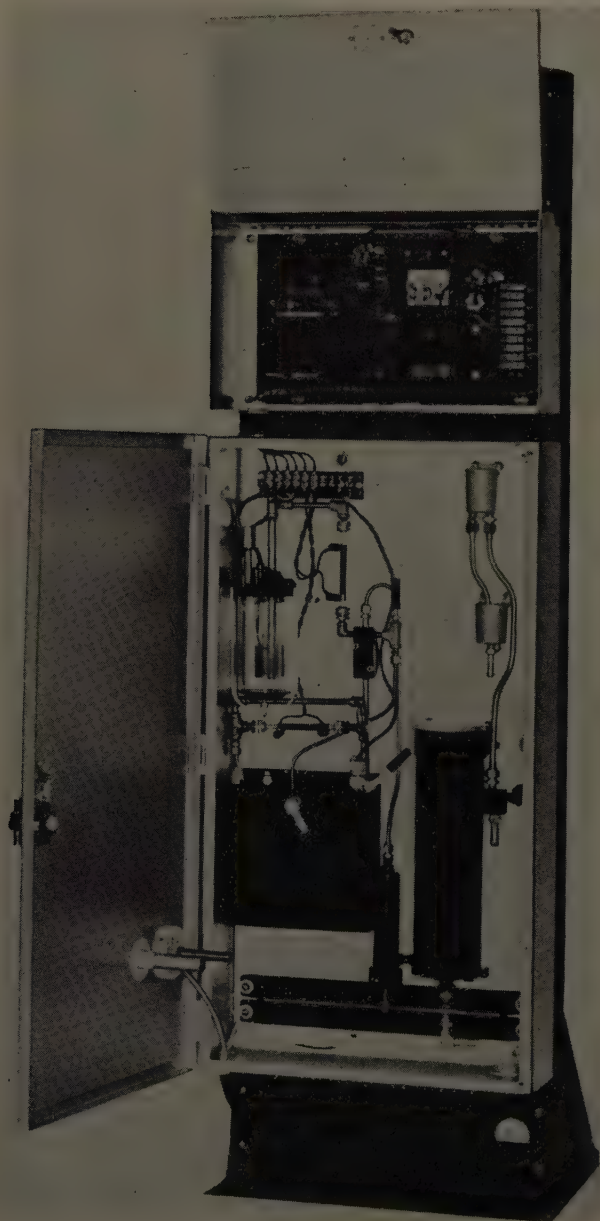


FIGURE 4. New type of dissolved oxygen recorder.

perform the analysis in the laboratory. Again, the need for relating the concentration of these materials with hour-to-hour and, sometimes, minute-to-minute changes in operating conditions has increased the need for continuous-recording colorimetric analyzers capable of performing automatically all the



FIGURE 5. Portable flow-type conductivity recorder.

steps of the analyses previously done by the chemist. One such instrument will be discussed in this paper.

### *Miscellaneous Tests*

Numerous special tests, formerly performed by an operator or station chemist when occasion demanded it, are gradually becoming automated. Such tests include the determination of the dew point of the hydrogen gas used for generator cooling, the determination of combustible gases in various areas, and the determination of the residual chlorine content in condenser cooling water after chlorination treatment to prevent the formation of slime on the tube surfaces. The oxygen content of flue gas commonly is determined automatically and recorded.

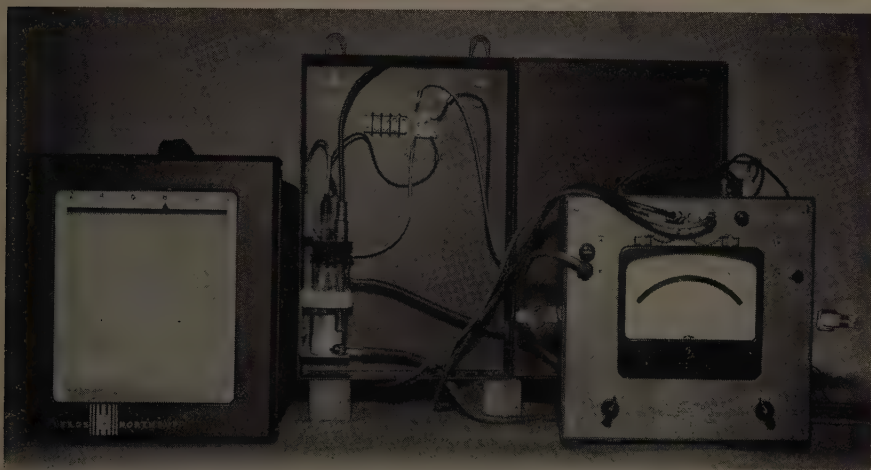


FIGURE 6. Portable flow-type pH recorder.

### *Portable Recording Instruments*

In many cases it is desirable to supplement permanently installed instruments with additional analyzers in order to gather additional data on some phase of plant operations. Portable recording instruments often are used for this purpose. FIGURE 5 shows a portable conductivity recorder complete with flow cells and electrodes. FIGURE 6 shows a portable pH instrument consisting of flow cells with electrodes, amplifier, and recorder.\*

### *Economic Aspects*

What criteria are used in the design of analytical instrumentation for modern electric stations?

First, let us explore the economic aspects of the question. A single unit, such as shown in FIGURE 1, may represent an investment of 50 to 100 million

\* Leeds and Northrup Company, Philadelphia, Pa.

dollars. This huge investment is justified by the fact that such units can deliver electricity to the distribution system at a considerably lower cost per kilowatt hour than can the older units. When the load normally carried by such a unit is transferred to older units, the differential cost, because of the lower efficiency of these units, may run as high as 10,000 dollars per day. Hence, the value of an instrument that can spot operating difficulties so that corrective action may be taken with a minimum of down time is readily apparent. The prevention of a single incident involving several hours' outage may result in the saving of repair costs and differential operating costs that may well exceed the cost of the instrument involved.

However, to perform this function successfully, the instrument must be on the job twenty-four hours a day, week after week. Instrument down time possibly could lead to serious operating difficulties in the station. Therefore, the dollars spent to ensure instrument reliability can be justified easily. An instrument, the price of which has been reduced to meet competition at the expense of performance and reliability, has no place in this field.

#### *Instrument Maintenance and Repair*

Unfortunately, no instrument can be expected to work forever without maintenance or repair. The speed and ease by which both may be accomplished are of prime importance. Ready access to all parts of the system, rapid testing and calibration procedures, and simplified replacement of defective parts, as well as complete operating and maintenance data, are factors that should be given considerable attention in the selection of the instrument to be used. A printed circuit board containing a large number of firmly attached components and anchored in an inaccessible location ultimately will cost many users far more than the amount saved by the manufacturer in using such a device. In this field there is no justification for miniaturization, transistorization, or the adoption of sales appeal devices, unless such will contribute to the performance and reliability of the instrument.

#### *Ambient Operating Conditions*

An instrument may perform well in an air-conditioned laboratory and be a dismal failure when exposed to the adverse environmental conditions often encountered in the station. An analytical instrument must be able to "put on overalls" and perform its assigned function satisfactorily under varying conditions of ambient temperature and humidity. It should be unaffected by atmospheric dust, vibration, or other conditions.

#### *Performance, Sensitivity, and Stability*

Last, but not least, one must consider the actual test work that the instrument is expected to perform. Here again, stringent requirements prevail. The low concentrations of the various materials to be tested require the use of instruments having exceptional sensitivity. In laboratory instrumentation extra sensitivity often can be achieved at the expense of long-term stability. This compromise is not permissible in plant operation. The instruments may be called upon to determine the concentration of materials present in the parts-



per-billion range in waters of a purity often exceeding that of the distilled water available in many laboratories. Under such circumstances one is working in an area of chemistry where platinum and polyethylene often are used to avoid contamination from glassware, and a strong stock solution used to make up standard solutions for calibration may have a strength of only 1 ppm.

### *Recent Developments*

The background and general requirements of analytical instrumentation have been discussed briefly. Now let us look at a few examples of recent developments in this field.

The Technicon AutoAnalyzer was originally conceived as a laboratory instrument for automatic colorimetric analysis. FIGURE 7 shows this instrument "dressed in overalls" and combined with a 10-point automatic sampling system and other miscellaneous accessories. This system automatically determines the phosphate concentration in boiler water from 10 different boilers once each hour and records the results. This information guides the plant operator in respect to the chemical treatment and blow-down required for each of the boilers. The instrument has been in operation at the Waterside Station of the Consolidated Edison Company for more than a year and has performed over 100,000 analyses since its installation. Several similar instruments currently are in use in other stations, recording silica, iron, chloride, and other substances.

For many years conductivity was the accepted method of determining steam purity. However, the overhead steam from modern boilers and evaporators is of such a high purity that conductivity measurements are no longer adequate. Recently, the determination of sodium in condensed steam samples by means of flame photometry has been used for this purpose. Thus far these analyses have been performed in the laboratory on grab samples or in the plant on flow samples with modified laboratory instruments that require close supervision by skilled technicians. FIGURE 8 shows a recording flame photometer developed by the Consolidated Edison Company and currently in manufacture.\* This instrument is capable of determining sodium concentrations as low as 0.1 ppb. It contains a built-in multipoint sampling system, an automatic self-calibration system, an automatic range-changing system, alarm systems, and other systems. This instrument is designed for continuous unattended operation. In addition to providing valuable information on the performance of boilers and evaporators, it will play a vital role in the operation of our nuclear power plant.<sup>1</sup> This is discussed below.

### *Analytical Instrumentation in a Nuclear Power Plant*

What about the future? A look at the problems in automatic analytical instrumentation at the Indian Point Nuclear Power Plant, currently under construction on the shore of the Hudson River at Buchanan, N. Y., will aid in answering this question.

An artist's conception of the completed plant is shown in FIGURE 9. This plant contains the usual components of a regular steam power plant, with the exception of a regular boiler. The boiler of the plant consists of four boiler-

\* Waters Associates, Framingham, Mass.

heat exchanger units, which are heated by water at high temperature and pressure circulating through a nuclear reactor. This reactor produces heat by the controlled fission of uranium<sup>235</sup>. It is of the breeder type, also producing fissionable uranium<sup>233</sup> from thorium<sup>232</sup>. A flow diagram of this plant is shown in FIGURE 10. Complex facilities for the removal and disposal of radioactive



FIGURE 7. Automatic 10-point colorimetric analyzer used for the determination of phosphate in boiler water.

wastes from the primary loop are necessary, as well as a water treatment plant of the most advanced design to produce ultrapure make-up water.<sup>2</sup>

All the basic analytical problems of a modern conventional plant are present in increased magnitude, in addition to the new problems associated with the



FIGURE 8. Ultrasensitive recording flame photometer used for the determination of trace quantities of sodium and boron.

determination of trace quantities of unusual materials in radioactive samples. The Indian Point plant will contain a complex sampling system that will feed samples from numerous points to a large number of analytical instruments. These instruments will include refined versions of the conventional *pH* and conductivity apparatus, special types of automatic colorimetric analyzers,



FIGURE 9. Artist's conception of the Indian Point Nuclear Power Plant of the Consolidated Edison Company of New York, Inc.

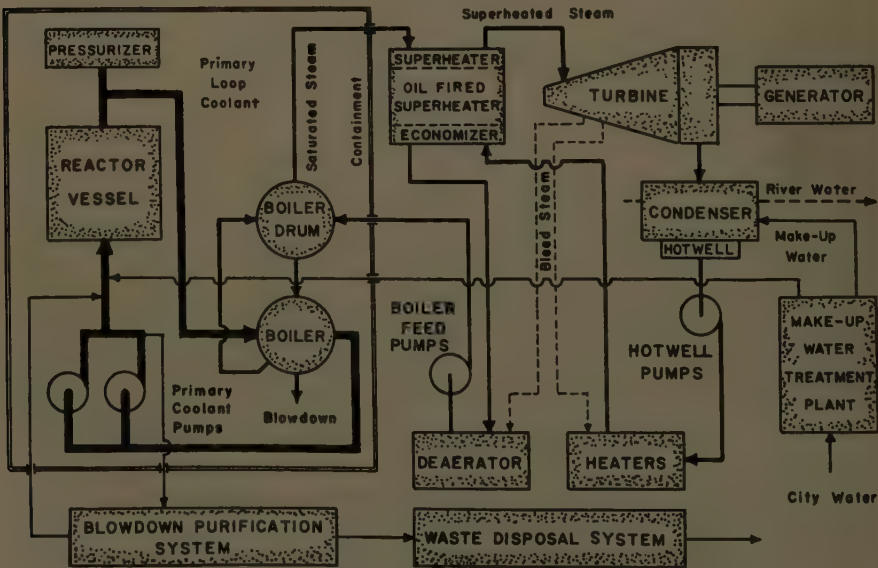


FIGURE 10. Indian Point Nuclear Power Plant. Simplified flow diagram.



oxygen analyzers of a new design, and dissolved hydrogen recorders. Automatic continuous sample concentrators will be used at various points to provide a continuous feed to automatic analyzers. These will be used when it becomes necessary to extend the effective sensitivity of an instrument by concentrating the sample up to one hundredfold. Gamma-ray spectrometry will be used to a considerable degree for the identification of radioactive isotopes.<sup>3</sup>

The recording flame photometer will play two vital roles in the instrumentation scheme of this plant. To prevent stress corrosion cracking of the austenitic stainless steel used in both piping and process equipment, the system must be kept free of chlorides. The most probable source of chlorides would be river water, entering the system through condenser leakage. Since this is essentially a closed system, any chloride leakage would concentrate rapidly in the boilers. Fortunately, sodium and chloride exist in the river water in relatively fixed ratios. An instrumentation system using these recording flame photometers will monitor the water in the condenser hot well continuously, and concentrations of sodium (with the accompanying chloride) in the order of a few parts per billion will actuate alarms and possibly isolate the section of the condenser containing the leak.

Boric acid will be added to the primary loop during start-up periods to control excess reactivity by means of neutron absorption in the boron. The boric acid later will be removed from the system, when equilibrium has been reached. During these periods the concentration of boric acid will be monitored by the same type of flame photometer, measuring boron instead of sodium.

### *Summary*

Automatic analytical instrumentation serves a vital and expanding role in the operation of a modern high-pressure steam electric generating station. The types of instruments required are many, and their applications are diversified. However, all these instruments have several common requirements. They must be reliable under all conditions of operation, they must be maintained and repaired easily, and they must be capable of accurately determining trace quantities of a variety of materials on a continuous basis and with freedom from the errors caused by such conditions as instrument drift, changes in ambient conditions, and loss of calibration.

The design, installation, and operation of such instrument systems present a great challenge both to the instrument manufacturer and to the user, but it is a task that can be accomplished successfully with a complete understanding of the problems involved and the close cooperation of all the parties concerned.

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## CONTINUOUS SAMPLING AND AUTOMATIC ANALYSIS FOR SILICA IN MODERN HIGH-PRESSURE BOILERS

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With the advent of higher boiler pressures for obtaining greater efficiency, chemical control has become increasingly important in preventing turbine blade deposits. As early as 1940 it was shown that more than 1700 pounds per square inch gauge (psig) steam can keep a quantity of salts in true solution.<sup>1</sup> These salts deposit upon the high and intermediate stages of the turbine, with a resultant loss of efficiencies and capacities in turbines operated in the 1800 to 2400 psig range.<sup>2</sup> Discontinuing the use of solid boiler feed-water treatment by the addition, for example, of sodium phosphate and sodium hydroxide appears to be effective in preventing this efficiency loss. Silica is still present, however, in the boiler water in a dissolved state, and it vaporizes and subsequently passes over into the turbine with the steam. This deposition is a hard coating of quartz or amorphous silica on the low-pressure sections of the turbine.

Vaporous silica carry-over was first encountered when boiler operating pressures were increased to about 1000 psig. The trend in the power industry today is toward larger capacity units and fewer scheduled turbine outages. As a result of this trend, much research has been done in an effort to explain the silica carry-over phenomenon.<sup>3-5</sup> This work has shown that a definite ratio exists between the amount of silica in the steam and the amount in the boiler water. The curves of FIGURE 1 show this relationship. It has been found through experience that, if there is 0.02 ppm silica in the steam, the amount of silica deposition on the turbine blades can be tolerated. With this 0.02 ppm silica taken as a maximal concentration for operating purposes, the curves readily show the maximal boiler water silica concentration for the pressure ranges of 400 to 3200 psig. For example, at a boiler operating station pressure of 2000 psig, about 1.0 ppm of silica in the boiler water will produce 0.02 ppm in the steam. With the same boiler water silica concentration and 2600 psig, about 0.06 ppm silica will be found in the steam.

The newest boilers installed in our system are designed to operate in the 2600-psig range; the curves of FIGURE 1 show that a concentration of only 0.3 ppm silica can be tolerated in the boiler water if the 0.02 ppm limit in the steam is not to be exceeded.

Silica control in boilers cannot be accomplished practically except by boiler blow-down accompanied by silica-free make-up water. Prior to the installation of the 2600-psig boilers, 1500 psig, which could maintain about 3.0 ppm silica without excessive carry-over, was the highest pressure in the system. This value usually was reached within 2 or 3 days following the initial boiler start-up by intermittent blowing down of the boilers. Once the silica reached an acceptable level, it normally could be maintained at or below this point with almost no further blow-downs while the boilers remained in service. These units are designed to operate with less than 0.5 per cent make-up to the boilers.

Before the start-up of the first of the 2600 psig boilers the question of the silica control point had to be decided, namely, to control the silica carry-over by measuring the silica quantity in the steam or to make use of the afore-

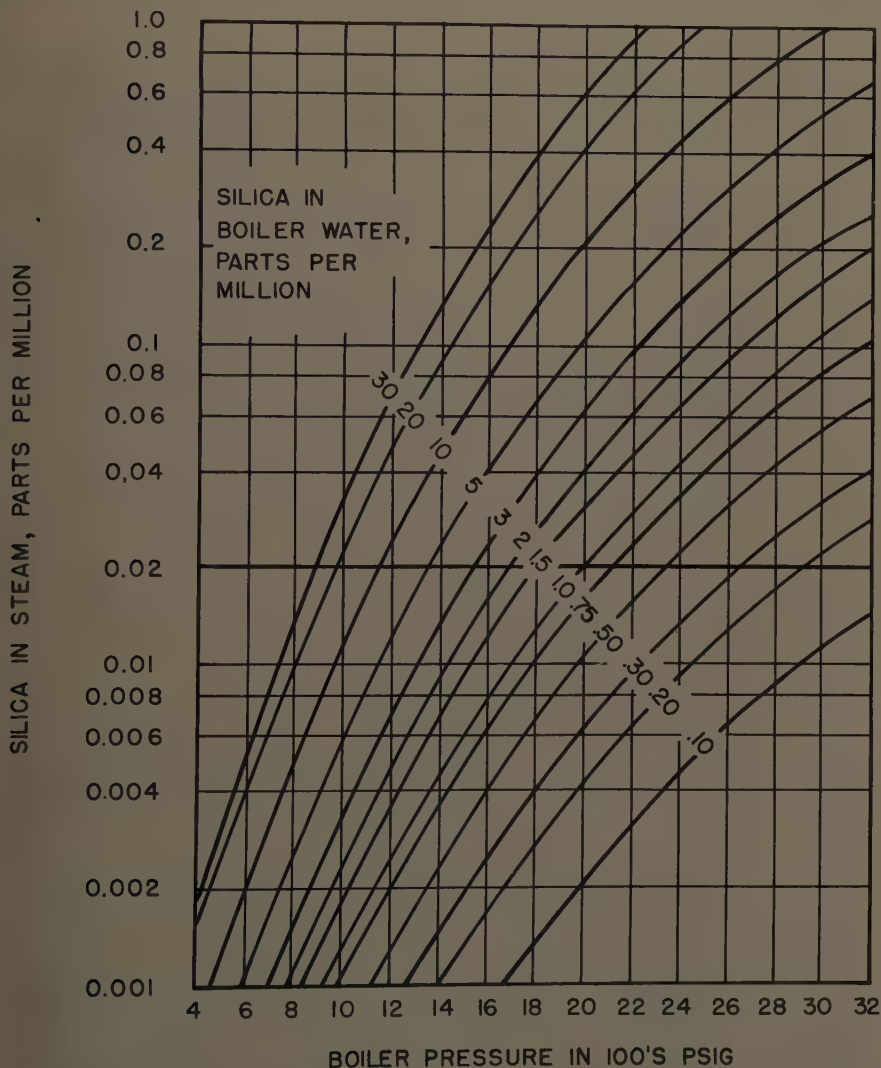


FIGURE 1. Calculated relationship of silica in boiler water, silica in steam, and boiler pressure. Reproduced by permission of A. B. Sisson, Commonwealth Edison Co., Chicago, Ill., from data of F. G. Straub, Univ. of Illinois.

mentioned curves, thereby measuring silica content of the boiler water and, by means of these curves, reading the quantity being carried over with the steam. It was decided to control the silica content of the steam by monitoring the boiler water, as sampling is easier since the higher magnitude of the result makes the determination less delicate and operating control is facilitated.<sup>6</sup>

As mentioned previously, the silica content of boiler water is always high at the start-up of any unit. The 2600-psig units were given a thorough cleaning in both the preboiler piping and the boiler itself. This preliminary cleaning aided in lowering the initial silica content; even with this cleaning, the silica was found to be as high as 7.0 ppm initially and, because of the maximum tolerable silica content at 2600 psig, it was necessary to analyze the boiler water hourly, 24 hours a day. The normal working period for personnel who do these analyses is 8:00 A.M. to 4:30 P.M., Monday through Friday. This would necessitate considerable rearrangement of personnel to control silica continuously.

Changes in the loading of a turbine vary the silica content in the boiler water. A decrease in loading is followed by an increase in silica content, while an increase in steam flow through the turbine causes a subsequent decrease in boiler water silica content.

By means of a curve developed from FIGURE 1 the boiler pressure was maintained at such a level that the silica passing into the steam would never exceed 0.02 ppm.

To facilitate 24-hour supervision of the silica content of the boiler, the use of a continuous automatic analyzer was deemed advisable. In addition, such factors as the time required for analysis and the greater reliability of an automated analysis were included among the reasons for continuous silica monitoring. About one-half hour was required for manual sampling and analysis. Continuous automatic monitoring requires much less analysis time and, again, greater reliability is achieved by minimizing the human element in both sampling and analysis. This continuous monitoring is accomplished by means of the AutoAnalyzer. The components of the system are shown in position in an enclosed cabinet in FIGURE 2. On the lower shelf are a proportioning pump and the mixing and time delay coils. On the upper shelf is the colorimeter, with the recorder housed directly above. A programmer for automatic sampling and standardization is inside the cabinet on the upper shelf. The basic principle of the AutoAnalyzer system and silica determination has been described in a paper by A. Ferrari and E. Catanzaro.<sup>7</sup>

FIGURE 3 shows the automated procedure used at present for the analysis. The sample stream enters the pump manifold at a rate of 2.5 ml./min. and is joined with a stream containing 0.6 ml./min. of a mixed reagent containing 1 per cent ammonium molybdate in 0.1 *N* sulfuric acid. The sample and reagent are air-segmented, mixed, and passed through a time delay coil. Air segmentation cleanses the system by the action of the air wiping the tubing; it also tends to average out any small variations that occur in the reaction. The mixer is simply a length of glass tubing bent into a coil shape and allowed to lie in a horizontal position; the mixing effect thus is created by the continuous inversion of the liquids passing through. The time delay coil is a continuous length of plastic tubing formed into a coil. Five per cent oxalic acid, 0.4 ml./min., is next introduced, again mixed, and then joined by 0.6 ml./min. of 1-amino-2-naphthol-4-sulfonic acid. After being mixed again, the combined streams are passed through another time delay coil and into the colorimeter. Absorbance is measured at 815 mμ. with a 10-mm. cuvette. The imbalance created in the electric circuit is amplified and recorded. Plastic tubing,  $\frac{1}{16}$



in. inside diameter, is used between all equipment for transporting samples and reagents.

A 3-position range expander ( $1\times$ ,  $2\times$ , and  $4\times$ ) is built into the recorder. By means of this any portion of the recorder range may be multiplied by a factor of 1, 2, or 4.



FIGURE 2. System components.

The calibration points of one analyzer operating with a range expansion of  $2\times$  are shown in TABLE 1. This  $2\times$  position is used to expand the 50 to 100 per cent portion of the recorder scale; this has been found to be the most prac-

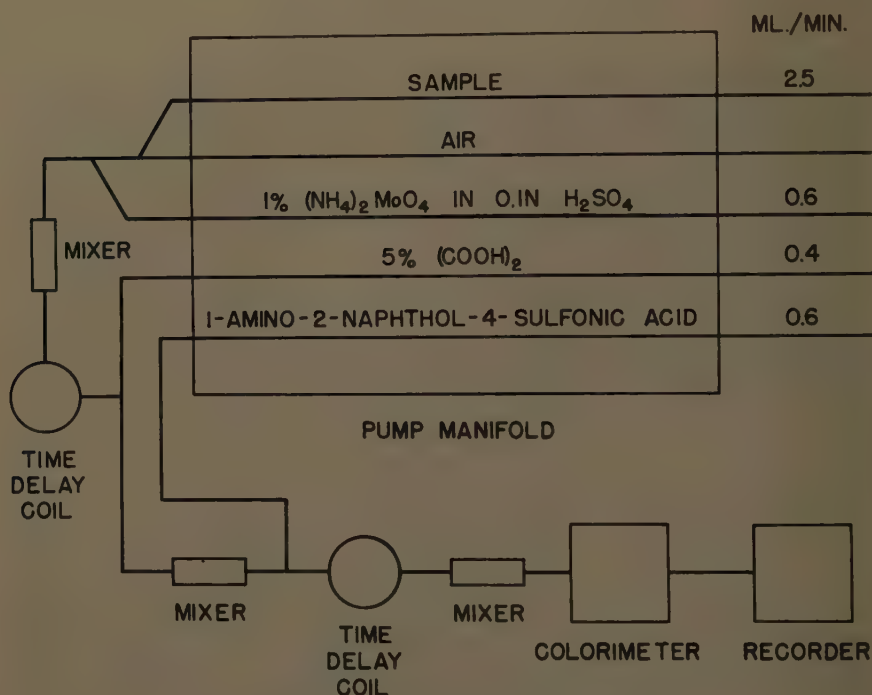


FIGURE 3. Automated silica procedure.

TABLE 1  
ANALYZER CALIBRATION,  $2\times$  RANGE EXPANSION

Silica (ppm)	Transmittance (%)
0	100
0.1	94.5
0.3	83.1
0.5	72.9
1.0	48.2
1.5	25.5
2.0	5.3

tical expansion, because the 0- to 2.0-ppm range is never exceeded except during some boiler start-up periods. Moreover, this range is acceptable for the normal silica content of 0 to 0.3 ppm. During start-up periods, if the silica content is greater than 2.0 ppm, the range expander may be changed to the  $1\times$  position when the exact silica concentrations are desired. In practice this procedure is seldom followed, as the silica-pressure relationship for operation

usually is within the range that may be controlled with the use of the 2X position within a short time period following a turbine start-up.

Finely divided, entrained material is present in boiler water and must be

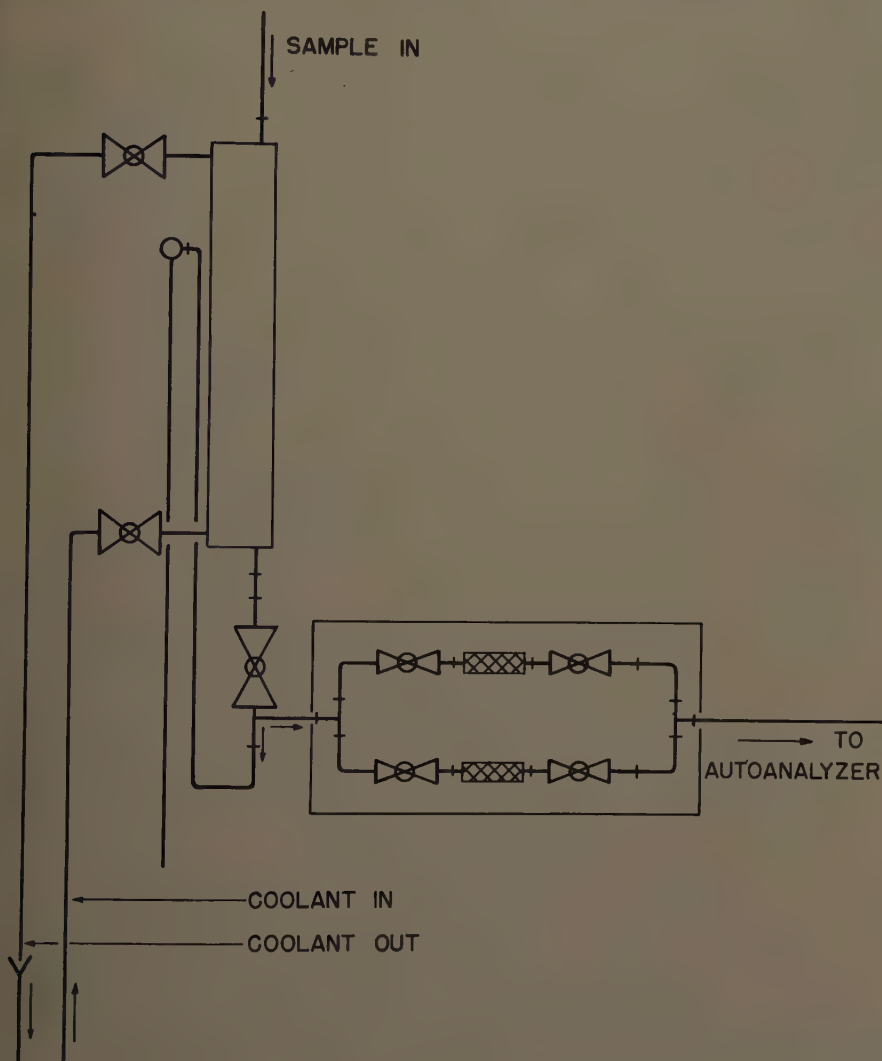


FIGURE 4. Sample cooling and filtering arrangement.

removed, before it enters the colorimeter, either by means of a separate module or by some other suitable means.

In the installation described, the undissolved material is removed by filtering the sample before it enters the AutoAnalyzer. FIGURE 4 shows a detail of the filtering arrangement of the boiler water sample. A sample of boiler water

is conducted through stainless steel tubing from a point on the boiler circulating pump discharge header through a cooler. A valve on the discharge of the cooler is used for controlling sample flow. Into the discharge side of the valve a T is connected, one side of which feeds the AutoAnalyzer through stainless steel filters with accompanying valves. These filter openings are approximately  $10\ \mu$  in diameter. Two filters are mounted in parallel to facilitate their cleaning without disturbing the sample flow, only one side of the parallel circuit being used at any one time. From here the sample flows to the analyzing equipment.

Stainless steel tubing is connected into the other outlet of the T and this tubing is so mounted that a head pressure of slightly more than 0.5 psig is maintained on the outlet side of the T to the analyzer. This slight head pressure is necessary to assure an uninterrupted sample flow to the analyzer at all times. At the uppermost part of this tubing a sight overflow with a vacuum breaker attachment is installed in order that the quantity of sample flow may be ascertained visually. The vacuum breaker attachment is necessary, because this excess sample water is not discarded, but put back into the feed-water system to be returned to the boiler. Owing to the design of the system, a siphon could be created that would prevent the flow of sample to the analyzer. A covered arrangement of the sight overflow is a safeguard against silica entering the system from the air at this point. With this filtering layout it is not necessary to filter the entire sample, which amounts to approximately 50 lb./hr., but only that portion (2.5 ml.) that is to be analyzed. It has been found that, following an initial period of operation, these filters need to be removed for cleaning on an average of only once per week.

Experience has shown that continuous monitoring entails problems that are mainly mechanical in nature. The chemistry of silica analysis is well known. In the analysis system designed for the AutoAnalyzer the concept of ratios certainly is sound, and for this reason no chemical problems were anticipated. While the process of analysis presented no problem, the first mechanical difficulty involved the life of the lamp used in the colorimeter. It was necessary to replace burned-out lamps at an average of once every 7 days. The minimum life was 4 days and the maximum, 10 days. To alleviate this difficulty, a 25-w. 0.25-ohm resistor was wired in series with the lamp. It was expected that this would increase the lamp life tenfold, with the output lumens being decreased about 50 per cent. This naturally decreased the sensitivity of the instrument but, fortunately, the range in which the analyses were being made was such that the decreased sensitivity did not adversely affect the results. With this resistance installed, the torque at the recorder balancing motor would be marginal when the recorder range expander was being used in the  $4\times$  position. In practice, the lamp life has been extended to 8 to 10 weeks.

Continuous automatic analysis necessarily increases the use of reagents. However, this should not be a deterrent to automation because, if an equal number of "hand samples" were taken, a proportionately large quantity of reagent would be used. The simplest means of decreasing the maintenance time required in changing reagents was by increasing the container capacity. Initially, 1-gal. plastic containers were supplied and, with the volumes used, it became necessary to replace reagents every second day. Two-gal. containers



were purchased to replace those originally in use and, to extend the reagent life further, the volumes of reagents were changed to correspond with those shown in FIGURE 3. The original volumes were 2 times those illustrated. These 2 alterations reduced the necessity of replacing reagents: once every 8 days was sufficient. When the reagent volumes were halved, the quantity of sample introduced was not changed, which tended to increase the sensitivity of the analysis. The original time for the analysis, from sampling to recording, was approximately 7 min. In the altered manifold arrangement it was increased to about 15 min. However, this increased time is not detrimental to boiler silica analysis work.

It has become necessary to keep a spare manifold at all times. The manifold is a system of polyvinyl chloride tubes placed upon a spring-loaded proportioning pump platen. Equally spaced rollers attached to stainless steel roller chains bear continuously on the tubes. The life was expected to be about 6 to 8 weeks. This has never been attained because the tubes flatten out and eventually crack open, owing to the continual bearing of the rollers on the tubes. Possible causes of the shortened manifold life, such as temperature, improper platen spring loading, and dirt, have been investigated, but no improvement has resulted. The polyvinyl chloride tubes are not affected by the reagents used in the analysis. It has become necessary to replace manifolds every second week in order to maintain continuous analysis. The maximum life obtained from any single manifold has been 3 weeks.

In steam plants it is often necessary to enclose equipment in cabinets or cubicles because of dirt and dust conditions and also to prevent damage by work forces in the immediate area.

The analysis instruments originally were designed for use in a laboratory on a table or bench. The arrangement is convenient for analyzing a series of samples, but for continuous monitoring an installation in the plant is required, because the laboratory is remote from the equipment to be monitored. Even with enclosed equipment, the location in the plant should be carefully selected. Besides a relatively clean and remote work area, such items as excessive ambient temperatures, accessibility for maintenance, sampling requirements, and availability of the recording for human supervision should be considered.

Recently we have placed in service, on two high-pressure boilers, sampling equipment and accessories for multipoint sampling. Equipment for automatic standardization of the analyzer is also installed, and a recorder is located far from the instrument. Only an indicating instrument was included at the analyzer itself.

When the original analyzer was installed, a portable unit was purchased that had none of the above-mentioned features. Thus an operator periodically had to inspect the silica recording and report it to the control room operator, who in turn had the control of the boiler blow-down. To simplify this arrangement, the remote recorder was installed in the plant control room, where it could be supervised continually by the control room operator. This original unit could sample only one boiler at a time. After a period of operation it was deemed desirable to know at all times the silica content of both the high-pressure units. This made 2-point sampling a necessity.

With remote recording it was necessary to know not only which sample

source was being recorded but also when the automatic standardization program was being recorded. This information was needed at both the indicating and recording instruments. It was needed at the recording instrument by the boiler operator and, at the indicating equipment, by personnel servicing the analyzer. Inasmuch as the boiler being sampled and the analysis recording do not necessarily correspond because of the time lag for analysis, it was also necessary to have an indication of the position of the sampling cycle at the indicating instrument. The former indications were handled by extra pens installed in the recorder mechanism. For the latter indications, two sets of lights were attached on the analyzer cubicle, one set indicating which stream was being analyzed and, the other, which stream was being sampled.

Control for the multipoint sampling is accomplished by the use of a programmer, which consists of a motor-driven shaft to which 5 cams are attached. These cams actuate switches that open or close the respective sample or standardization valves and operate the signals at the remote and indicating recorders for the sampling and recording indications. The sampling program has been set up to allow for a 15-min. sample stream from boiler A, followed by a 1-min. flush. A 15-min. sample stream is then introduced from boiler B, followed by a  $6\frac{1}{2}$ -min. combination flush, automatic standardization, and subsequent flush, for a total cycle time of  $37\frac{1}{2}$ -min. This cycle repeats itself continuously, thus allowing for 2 analysis periods per hour for each boiler. As the changes in boiler silica concentrations are gradual, any such changes can be noted in time to make adjustments in boiler blow-downs and operating pressures.

If at any time it is desirable to monitor one boiler continuously—for example, in boiler start-up periods or when one boiler is out of service—the programmer unit may be altered. A 4-position switch is located on the front of the programmer and it is necessary to change only the position of this switch to satisfy continuous single-boiler monitoring.

By means of the programmer unit it is also possible to send continuously a standard or known silica concentration stream through the analyzer.

The automatic standardization takes place for a period of about  $1\frac{1}{2}$  min. during the  $6\frac{1}{2}$ -min. interval between the analyzing of boilers A and B. Following a flushing period of  $1\frac{1}{2}$  min., the standardization relay is cycled on and off 10 times. The recorder stylus is returned to a predetermined setting by means of a potentiometer placed in the circuit during each cycle.

Silica-free water, obtained from a 4-bed demineralizer, is used for flushing periods between samples and also for automatic standardization. Any known silica concentration within the expanded range may be used for this purpose, but the use of silica-free water eliminates the possibility of improper standards and saves manpower time required for preparation.

Demineralization is the process by which make-up water for these units is prepared. Naturally, a very low silica content is necessary. Continual surveys of the demineralizer effluent for soluble silica are possible by means of continuous automatic analysis. In this way silica-free water for make-up purposes is guaranteed. The amount of the soluble silica in the demineralizer effluent used for the 2600-psig boilers is 0.005 ppm in an average demineralizer

run. With this value obtained for soluble silica, tests have shown that the total silica, including both soluble and colloidal, averages about 0.007 ppm. By the use of continuous analysis this process can be observed and pure make-up water guaranteed. It is planned to include this sample in the automatic analyzer in the near future.

For high-pressure boilers in which silica concentrations must be kept very low the use of continuous automatic analysis has proved to be advantageous to the operator in the steam power plant. During periods of start-up and load changes the silica concentrations are high and variable; continuous analysis offers a convenient and reliable control at these times. In power plants, cabinets should be employed for protection of the equipment. Extra features such as automatic standardization, remote recording and, when it is desirable to monitor more than one point, automatic sample programming are other aids in controlling silica effectively.

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# THE DESIGN OF AUTOMATIC TRACE ANALYZERS

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In the preceding articles a number of interesting applications of automatic chemical analyzers have been described. These developments, for the most part, were designed around a final colorimetric measurement. Many different terminal methods are possible, of course; however, this paper will be limited to descriptions of a few electrochemical methods as applied to continuous chemical trace analyzers.

## *Hydrogen Ion Analyzers*

Perhaps the glass electrode pH meter can be considered as the original instrument for continuous analysis. This glass- and reference-electrode combination, in conjunction with a suitable electronic amplifier, constitutes an analytical instrument that is specific not only for hydrogen ions but also, owing to its extreme sensitivity, for trace quantities of hydrogen ion. It is probably safe to say that more pH instruments are being used in continuous applications today than the total of all other automatic or continuous analysis units. Many of these pH systems utilize reagent addition to make the measurement specific for a single compound.

## *Specific Ion Analyzers*

The principle governing the operation of the glass electrode in hydrogen ion determinations can be applied effectively to other types of specific electrodes. Much effort has been expended in the past few years on the development of special electrode systems to give direct, essentially instantaneous measurements of the concentration of specific ions as well as specific compounds. Electrodes developed to date for this purpose are sensitive to sodium, potassium, calcium, silver, sulfide, chloride, and iodide ions. Moreover, special electrodes have been developed for measuring both oxygen and carbon dioxide.

In many instances, these electrodes operate with an accuracy of better than  $\pm 5$  per cent of the amount of material present over a concentration range of 1 to 10,000 ppm, or four decades of concentration.

## *Chloride Ion Analyzers*

The chloride electrode may be considered as typical of these specific electrode detectors.<sup>1</sup> FIGURE 1 shows the construction details of the silver-silver chloride billet electrode. The sensing portion is constructed of a sintered or pressed mass of finely divided silver and silver chloride. This mixture is homogenous in the electrode and consequently provides a highly reproducible signal throughout its extremely long life. In use, its response closely approximates the Nernst equation, providing 59 mv per decade of concentration change down to the concentration equaling the solubility product of silver chloride. FIGURE 2 represents the response of the electrode system over a four-decade range. Since the Nernst equation contains a temperature term, all measurements must be made at a constant temperature or, alternatively, the amplifier portion of the ana-



lyzer must be designed to compensate automatically for temperature changes in the sample stream.

The chloride ion electrode has many industrial applications. For example, it can be used in measuring the degree of pollution of river and surface waters. The chloride ion concentration serves as an effective index of pollution, since chloride is common to both industrial and sanitary wastes. Therefore, the dis-



FIGURE 1. Construction details of the silver-silver chloride pressed billet electrode used for measuring chloride ion concentrations.

charge limits or the degree of contamination can be estimated from the chloride ion concentration of the surface water.

Other direct applications include the measurement of chloride ions in nuclear power plants and in the boiler water of seaside power stations using salt water cooling. Similarly, the chloride analyzer can be used to detect salt water intrusion into the water table along coastal areas. Indirect applications include the determination of trace quantities of chlorinated organic compounds such as insecticides and like compounds in the atmosphere, in plant tissue, and in prepared food products.<sup>2</sup>

The silver-silver chloride electrode has been used also as a specific detector in gas chromatography. In this application the sample, after passing through the chromatograph, is drawn continuously at a constant rate through a combustion furnace where the bound chloride is released. The vapor from the furnace is scrubbed by a constant-flow water stream, and the dissolved chloride is measured with the silver-silver chloride electrode.

### *Oxygen Analyzers*

Other types of electrodes have been developed that are sensitive to specific compounds such as oxygen and carbon dioxide.<sup>3,4</sup> FIGURE 3 shows diagram-

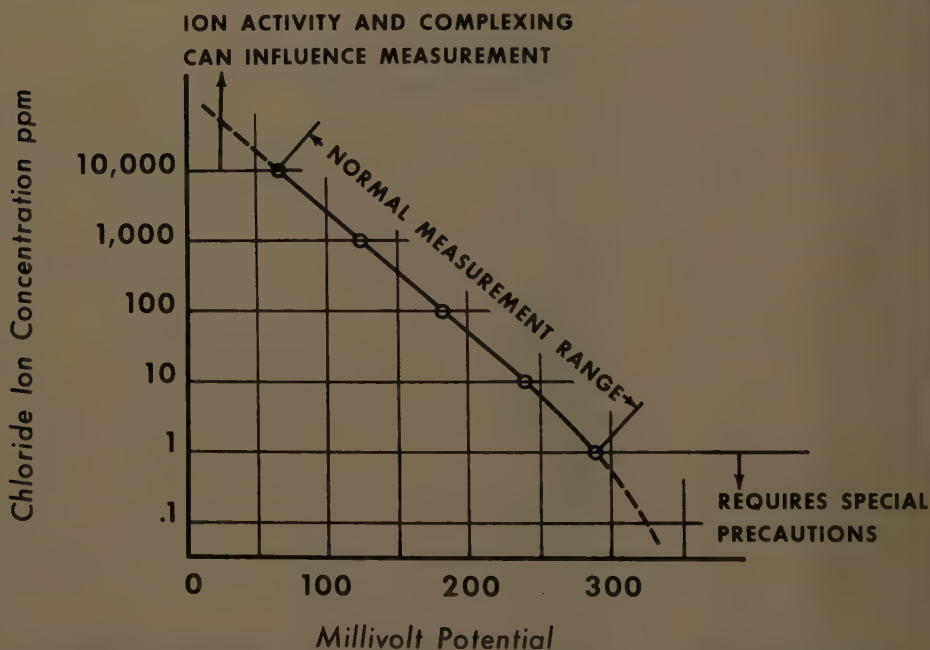


FIGURE 2. Response curve of the chloride ion electrode to a four-decade change in chloride ion concentration.

matically the oxygen electrode that operates on a polarographic principle. The system, as shown in FIGURE 3, is quite simple. It consists of a probe containing a platinum cathode, a silver anode, and a gas-permeable membrane arranged in a unique manner. The platinum surface is pressed firmly against the inside of the membrane and is connected electrolytically to the silver anode through the chloride-containing electrolyte in the cell. Dilute potassium chloride solution has been found to be a convenient electrolyte. The potential applied to the cell (about 0.6 v) is too low to cause discharge of hydrogen and, in the absence of gases reducible at this voltage, the cell polarizes. However, if a reducible gas is present in those gases passing through the membrane, a current flows in the cell in proportion to the partial pressure of that gas. Oxygen is the most common gas meeting these requirements. These electrodes

have been made microsize for insertion into the cubital vein of a patient in direct measurements of  $O_2$  tension in the blood. Typical uses of electrodes of this type, in addition to the direct measurement of  $O_2$ , are determinations

### OXYGEN ELECTRODE

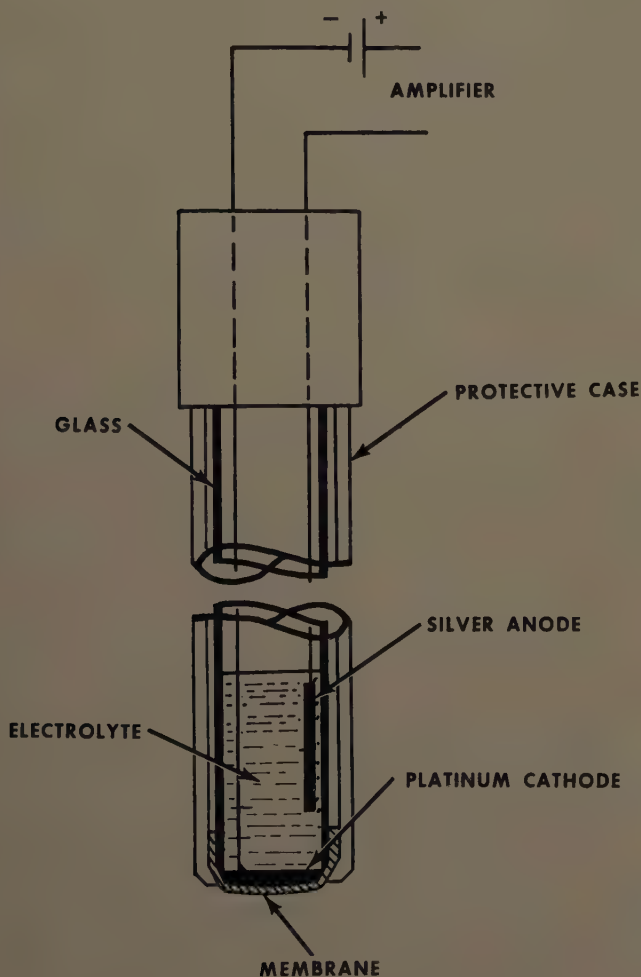


FIGURE 3. Schematic diagram of oxygen electrode.

of increases or decreases in  $O_2$  or  $CO_2$  concentration in samples treated with heat, light, or chemical energy.

#### *Dissolved Oxygen Analyzers*

Another extremely sensitive type of electrochemical measurement is that of the electric conductance of solutions.<sup>5,6</sup> Very simple measurements of this

sort have been in use for years in power and related industries to provide data on the quality of water. In this application the determination is essentially nonspecific.

It is quite possible, however, to devise methods based on this principle that will make feasible the design of highly specific and extremely sensitive analyzers. Basically, this can be accomplished by making two measurements on a sample, one before and one after a chemical or physical chemical reaction. The reaction in this case is chosen to be specific for the constituent to be measured. Hence, the change in conductance is proportional to the concentration of the substance in question.

For example, this principle is used in the design of an extremely sensitive trace analyzer, the dissolved oxygen analyzer (FIGURE 4). This instrument

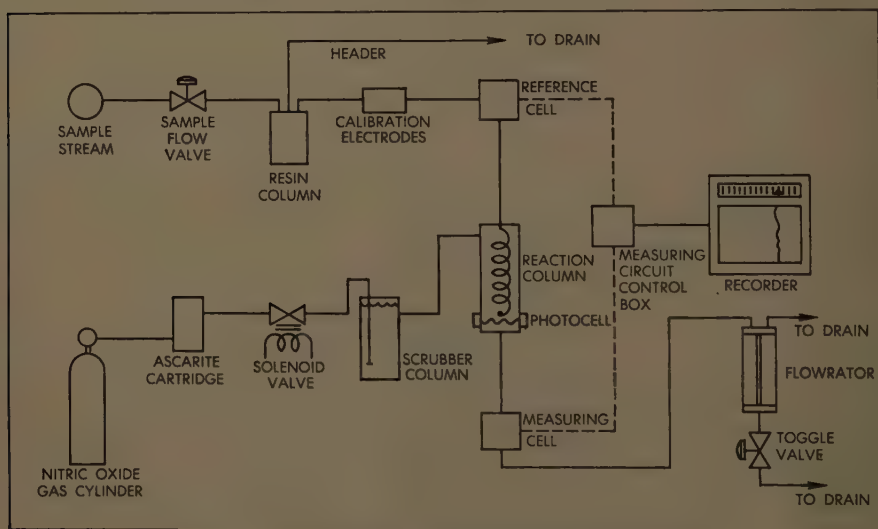
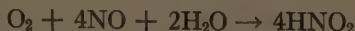


FIGURE 4. Flow diagram of the dissolved-oxygen analyzer.

was designed to measure oxygen in boiler feed water in the range of 0 to 25 or 0 to 100 ppb with a sensitivity of  $\pm 0.5$  ppb and a measurement accuracy of  $\pm 5$  per cent. The analysis is based upon the reaction of nitric oxide with oxygen:



This reaction goes to completion very rapidly and is not reversible under the conditions of analysis. Nitric oxide is an ideal reagent in that it does not react with water and does not ionize: hence, it does not contribute to the conductance of the sample. The reaction product of NO with  $\text{O}_2$  does ionize and therefore increases the conductance of the sample in proportion to the concentration of the  $\text{O}_2$  present. Other gases normally present in water do not interfere; however, extraneous ionic materials in the sample will reduce the sensitivity. These substances are removed by passage of the sample through a cation exchange resin prior to measurement.



The operation of the instrument is straightforward. After being routed through the cation exchange resin, the sample passes through a reference conductivity cell where a signal proportional to the initial conductivity of the sample is developed. It then passes through a reaction chamber and is placed in contact with nitric oxide that has been purified by scrubbing with trisodium phosphate to remove higher oxides of nitrogen and other acidic materials. The reaction chamber is designed to allow maximum contact of the sample with the nitric oxide gas. The reaction products remain dissolved in the water and are measured in the second conductivity cell. A measuring circuit provides an AC voltage to the conductivity cells, compares their resistances (which are inversely proportional to their conductivities), and rectifies the difference signal to a DC voltage which drives a potentiometric recorder.

To circumvent the difficulties in checking the accuracy of this technique by conventional laboratory methods, a calibrator has been built into the sensing portion of the instrument. This calibrator functions by providing exact concentrations of oxygen by electrolysis. The amount of water electrolyzed or  $O_2$  formed is calculated from the current flow by Faraday's Law.

#### *Mercaptan Analyzer*

A second instrument based on the differential conductance technique is designed to measure the concentration of mercaptans in refinery and natural streams (FIGURE 5). In intrastate distribution and interstate transport of natural gas, odorants must be added to allow sensory detection of leakage. Mercaptans are used for this purpose. Too great a concentration of odorants gives rise to user complaints or fatigue effects, while concentrations too low for sensory detection are equally hazardous.

If the odorants were stable they could be added simply at known concentration levels at the source. However, they are not stable nor is their instability predictable. The mercaptans degrade at various rates to sulfides, disulfides, sulfoxides, and sulfones. Thus a continuous measurement is required.

One of the outstanding features of mercaptans is their readiness to react with certain heavy metals and heavy metal salts to form insoluble products. Silver mercaptide, for example, is very readily formed and is so insoluble that it precipitates when a strong acid is the other reaction product. The estimated solubility product of silver mercaptide is  $10^{-16}$ .

Stoichiometrically, in the silver mercaptide reaction a hydrogen ion is formed for each mercaptan ion that reacts with a silver ion. In view of the relative mobilities of hydrogen to silver ions, there should be a great increase in the conductivity of a dilute solution of silver nitrate following the reaction. Moreover, this increase in conductivity should be proportional to the hydrogen ions formed and hence to the mercaptan content of the gas.

Unfortunately, interference was encountered in the form of carbon dioxide in the sample.  $CO_2$  and water dissociated and an equilibrium between carbonate-bicarbonate ions and hydrogen ions was established. There appeared to be two ways to circumvent this interference. The first, removing the  $CO_2$ , was not successful since the  $CO_2$  could not be removed completely without removing some of the mercaptan. The second way was successful. The sample is divided into two streams, a measuring stream and a reference stream. The

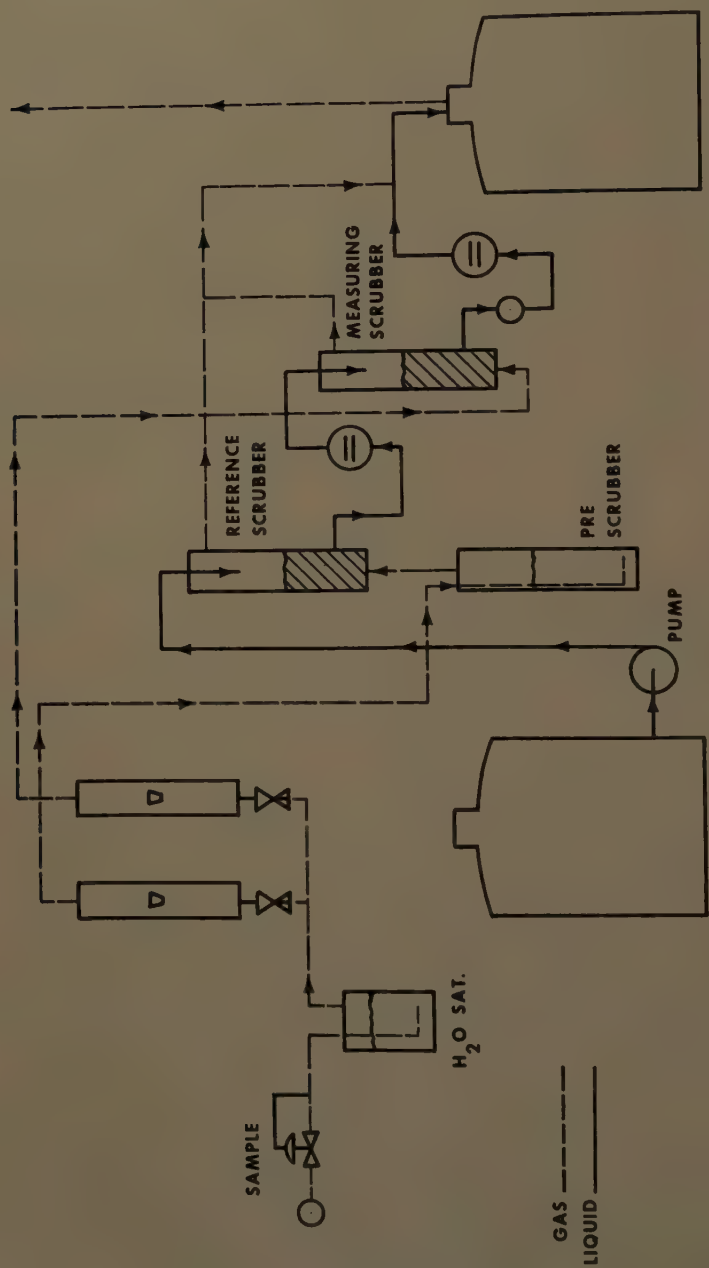


FIGURE 5. Flow diagram of the continuous mercaptan analyzer.

mercaptan can be removed completely from the reference stream without materially affecting the  $\text{CO}_2$  concentration. This reference gas then is scrubbed by dilute silver nitrate solution which absorbs  $\text{CO}_2$  and releases hydrogen ions in a concentration and hence a conductance dependent upon the partial pressure of  $\text{CO}_2$ .

The gas to be measured passes through a similar scrubber. In this case, however, the total conductance of the solution consists of a portion arising from the  $\text{CO}_2$ , which equals that of the reference, and a portion arising from the mercaptan concentration. Thus, the net difference in conductance between the reference and measuring cells is a direct measure of the mercaptan content of the flow-controlled stream.

Mercaptan concentrations ranging from 1 to 10 ppm (full scale) can be measured with a reproducibility of 0.1 ppm (0.00625 grain S/100 cu. ft.) with a  $\text{CO}_2$  variation of 0 to 1 per cent.

### *Moisture Analyzers*

The moisture content of many gases and liquids over the range of 1 to more than 1000 ppm can be measured as the current required to decompose the moisture absorbed on a film of  $\text{P}_2\text{O}_5$ . This technique was developed by F. A. Keidel.<sup>7</sup> The sensing element consists of two spirally wound platinum wire electrodes embedded in the inner surface of a plastic capillary.  $\text{P}_2\text{O}_5$  is deposited on the spiral area between these two electrodes. As the sample passes through the capillary, the water is absorbed by the  $\text{P}_2\text{O}_5$ , which then becomes conductive. When a current is passed through the electrodes the water is electrolyzed, and at equilibrium the current reading is a very accurate measure of the moisture content of a fixed flow of sample.

The major application of this device has been the direct measurement of moisture. The element has been used also as an extremely sensitive detector in gas chromatography and other areas where hydrocarbons and other compounds containing hydrogen are oxidized catalytically to form water.

### *Galvanic Cell Oxygen Analyzers*

Another electrochemical method is based on the principle of the galvanic cell.<sup>8,9</sup> Trace quantities of oxygen in gases can be measured accurately with a galvanic cell consisting of a lead anode and a silver cathode partially immersed in an alkaline electrolyte.

In the presence of oxygen an electrochemical reaction occurs at the interface of the cathode whereby the oxygen is converted into hydroxyl ions. This reaction results in the flow of electric current. The magnitude of this current is determined almost entirely by the oxygen content of the gas sample; more strictly, it is proportional to the partial pressure of oxygen in the gas that surrounds the electrodes. In the absence of oxygen no reaction takes place within the cell and no current flows.

The net result of this electrochemical reaction is the consumption of oxygen and the dissolution of the anode lead to plumbite ions. Electrolyte is not consumed in the cell reactions. However, water is lost from the cell if dry samples are passed through. This effect is eliminated by humidifying the gas

in the scrubber before it enters the analysis cell. In addition to humidifying the gas, the scrubber removes impurities. The water lost from the scrubber is replaced automatically with pure water from a water reservoir.

The analyzer can be used on most hydrocarbons (for example, ethylene and

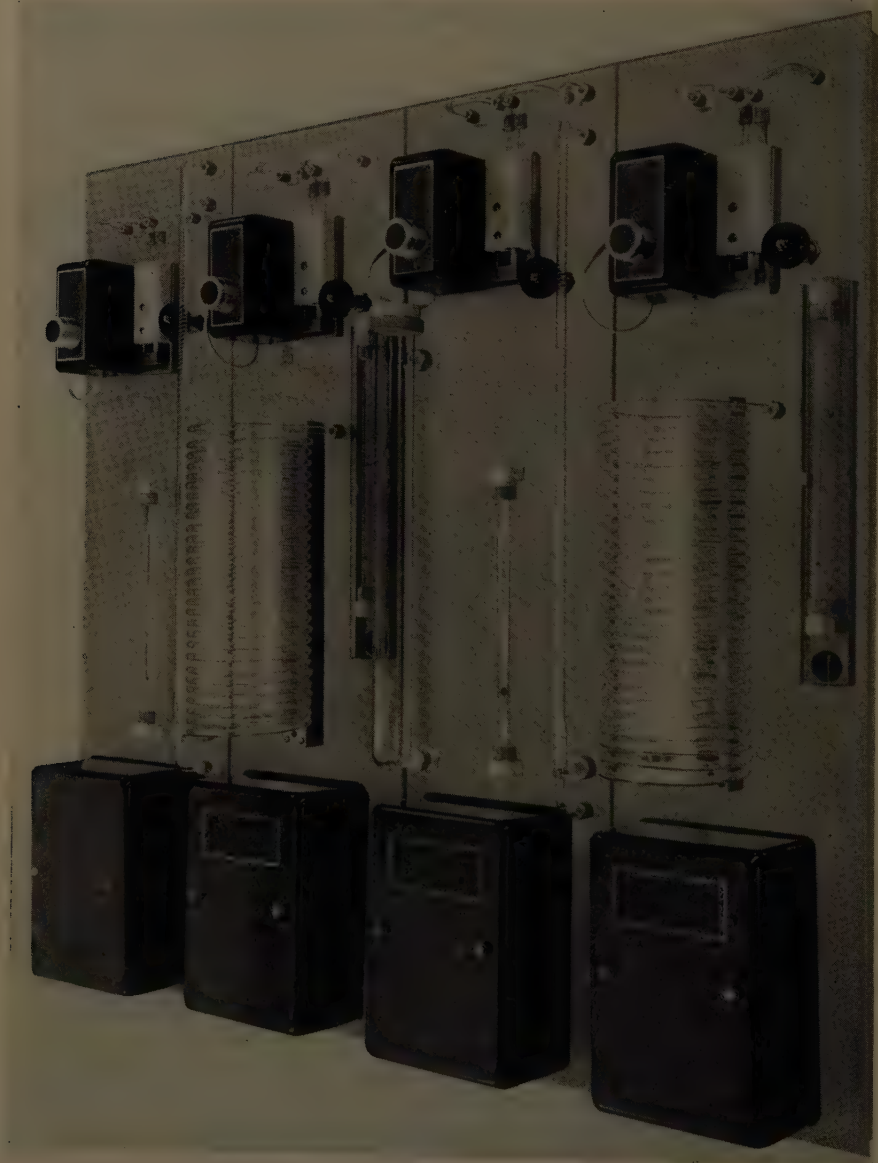


FIGURE 6. Positive displacement pumps designed for continuous analyzers incorporated into an air-pollution monitor.



butadiene) and on noncondensable, inert gases such as nitrogen, hydrogen, helium, argon, or mixtures of these gases.

### *Pumps for Automatic Analyzers*

One of the most critical factors in the design of automatic trace analyzers based on a reagent addition system is the accurate metering of small flows of reagents. Precision pumps for heavy industrial use have been available for many years; however, these are usually not satisfactory for use in a continuous analyzer, owing to their size and cost. Small bellows-type pumps are available, but these are usually not suitable for precise measurement owing to leakage past the gravity check valves. A positive displacement pump with mechanically activated valves has been designed specifically for the precise delivery of small flows. FIGURE 6 shows these pumps in use with an air pollution monitor.

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## PANEL DISCUSSION: PROBLEMS ON AUTOMATION OF CHEMICAL ANALYSIS AND INDUSTRIAL PROCESS

Jacques M. Kelly, *Chairman*

JACQUES M. KELLY (*Chas. Pfizer & Co., Inc., Brooklyn, N. Y.*): The concept of automation in chemical analysis cannot be considered a creation of the last decade. As early as 1914, semiautomatic titrators of individual designs were in satisfactory service, and a number of automatic systems for measuring  $pH$  with feedback mechanism were in use in process control. Developments until the latter 1940's continued to deal mainly with the automatic measurement of one particular physical property of a discrete sample or the analysis of a chemical constituent of a sample by physical means. The dramatic advances in electronics and servo devices during World War II spurred this development and, by the war's end, many ingenious automated process control systems were on stream in the petroleum and chemical process industry and in nuclear energy installations.

In the meantime, the importance of the application of classic chemical analysis in industry, medicine, and all aspects of research became more widely recognized, and the analytical chemist gradually became confronted with "mass production" problems. Because of economic considerations, this evolution gave rise to a need for the routine analytical technician who, because of his obvious limitations in training and experience, was less reliable than the professional analyst. As in most conditions of the natural order, progress comes as the result of an obvious necessity. Such is the case with automation in analytical chemistry.

We have seen from current literature how the automatic measurement of a specific physical property on a programmed basis now is accomplished easily. For example, continuous infrared spectrophotometry or refractometry controls many distillation processes; ultraviolet spectrophotometry, coulometry, and a variety of other electrical methods have wide application. This monograph highlights to a fascinating extent the recent developments in automation of the classic or "stepwise" chemical analysis, which requires a number of manipulations. Numerous systems have been described that automatically carry out the steps involved: sampling, purification and separation, addition of reagents, chemical reaction, analytical measurement, recording, controlling, and feedback.

We have seen clearly that the alliance of the analytical chemist and his colleagues in such fields as engineering and physics has firmly laid the foundations for progress in automated analytical chemistry. Of equal importance, also, is the automation "state of mind" in these areas, now a definite reality. The combination will beget further progress that will challenge the imagination of the entire scientific community.

This panel discussion inquires into "Problems on Automation of Chemical Analysis and Industrial Processes." Views of a number of representatives from universities, government, and industry are presented. In these remarks may be sensed the enthusiasm of accepted challenge; one may learn of things

to come in the clear delineations of problems, and astute approaches to solutions are indicated.

JOHN B. FREEMAN (*Bethlehem Steel Co., Bethlehem, Pa*): One of my jobs in the Research Department of Bethlehem Steel Company is to investigate the use of automatic chemical analyzers for the determination of cyanide and phenol in industrial waste effluents. We should like to use automatic chemical analyzers there for the continuous monitoring of plant waste effluents for cyanide and phenol content. We could then obtain information that would be most useful in our continuous and vigorous program to prevent stream pollution.

During the past year we conducted an investigation of commercial analyzers on the market and learned that there are two or three high-quality instruments that can determine the cyanide and phenol content of aqueous media under favorable conditions. Each instrument, we found, has both commendable features and admitted limitations. It is true that the instruments will determine cyanide and phenol in water solutions free from interferences that affect colorimetric readings. In practice, however, the effluents to be analyzed will contain other pollutants that obstruct the colorimetric determination of cyanide and phenol. Thus, unfortunately, prescribed methods of determining cyanides and phenols in waste effluents involve specific procedures that transcend the capabilities of these instruments as presently sold. This fact, however, in no way detracts from the excellence of the instruments, but merely emphasizes the difficulties found when analytical methods for cyanides and phenols are automated.

The features in these methods that are especially difficult to automate are those involving preliminary sample treatment for removal of such substances as sulfides, oils, and metal ions that might interfere with some stage of subsequent color development. Also, in cyanide analysis it is necessary to decompose complex cyanide compounds into simple cyanide ( $\text{CN}^-$ ), which can be quantitatively determined. This, of course, means that an intricate sample step preceding the use of the automatic analyzer is necessary.

To the best of my knowledge, there is no commercial analyzer on the market that can perform some of the more common manipulations generally associated with chemical analyses, such as distillation, solvent extraction, and filtration. Instrumentation to accomplish some of these operations is necessary in automating a good method of determining cyanides in waste effluents. At present, we can perform excellent analyses of cyanides in samples free from interferences by means of an automatic analyzer. Determinations of as little as 25 parts per billion (ppb) can be made with good precision. However, for practical use in the field, it will be necessary to devise an automatic apparatus that can do the following: (1) pump a raw sample from the effluent stream to a device that can measure a precise quantity for analysis; (2) distill the measured sample in a high-speed still in the presence of a mild acid such as tartaric acid; (3) collect the distillate in an approximate volume of mild caustic and then dilute to a precise final volume; and (4) feed the purified final volume to the automatic analyzer for  $\text{CN}^-$  determination.

Until such time as the above steps are automated, we shall be seriously handicapped in the use of automatic analyzers for cyanide determination.

The automatic analysis of phenols appears to be simpler than that of cyanide, provided the lower limit of phenol concentration in aqueous media is 500 ppb or more. A method of phenol analysis has been automated successfully on an automatic analyzer for raw water samples with a phenol content of 500 to 7000 ppb. Contents of less than 500 ppb would require additional sample treatment such as sample distillation and chloroform extraction, operational steps that at present are beyond the capability of available instruments.

In conclusion, let me summarize the problems associated with cyanide and phenol analyses in this way: automatic analyzers for cyanide and phenol now exist, but there is need of more sophisticated instrumentation that can automatically perform the simple manual procedures of distillation, measuring, solvent extraction and, perhaps, filtration. The use of automatic analyzers could be expanded if such additional facilities were available as accessory items for the instruments. These features will undoubtedly appear as the art of automation progresses, and we hope that we will contribute in some way to this advancement.

ELLIOT H. BAUM (*Refined Syrups & Sugars, Inc., Yonkers, N. Y.*): To keep up with increased public demand, science and technology have made available to the manufacturer equipment that helps him to multiply output and enhance sales capacity. The modern industrialist is continually faced with the need of making changes in his mode of production that afford a minimum of cost and do a job more efficiently. However, production capacity is only one factor of importance to the manufacturer. The obvious advantage to the consumer of a free enterprise is that two or more companies provide the same products. Competition leads the businessman to be conscious of the quality of his goods. In the food industry the quality control laboratory is of vital importance. Up to the present time it has been the responsibility of individuals in the control laboratory to maintain quality standards, usually by long and tedious procedures of periodic sampling throughout the plant and manual analysis of each sample.

In recent years we have seen the gradual advent of automatic analytical systems. In most instances we can readily see the advantages of automation as a time- and labor-saving device, but of added significance is the increased sensitivity and abundance of reliable data that may be obtained.

In our refinery there are a number of locations where automatic analytical instrumentation is desirable. For example, an on-stream automatic chemical analyzer can provide for continuous analysis of sugar concentration in boiler feed water and, if desired, control can also be achieved. We now employ the AutoAnalyzer for this purpose. Since process conditions are rapidly changing, only an automatic and continuous operating piece of equipment can provide accurate data on sugar losses or contamination. Continuous chemical analysis of Monobed and cation exchange resin influents and effluents for calcium, magnesium, sodium, and potassium would be of great value in estimating the efficiencies of the various resins. A method of determining total invert concentration in our liquid invert finished products is much needed. I could dis-



cuss at least a dozen other worthwhile applications that could be made in our refinery.

The importance of automatic chemical analysis in the sugar industry is becoming apparent, and its realization awaits the efforts and leadership of progressive individuals.

IRA L. SHANNON (*School of Aviation Medicine, Air University, United States Air Force, Randolph AFB, Texas*): It has long been our desire that the clinical chemist take a long and respectful look at parotid fluid as a new and challenging analytical fluid, and that he realize that, in some instances, this fluid offers distinct advantages over blood and urine. Chief among these advantages are the ease of collection and the possibility of continuous sampling without altering the subject's physiological state by the collection procedure, the latter being of primary importance in adrenocortical steroid studies.

It seems quite clear that urea levels in parotid fluid are quite as valid as those in blood. In addition to the studies previously discussed, we have devised a parotid fluid-collecting system complete with an overflow outlet for excess fluid, and with this device we have connected our subjects directly to the AutoAnalyzer. Parotid fluid urea levels thus are monitored continuously as alterations in blood levels are experimentally induced. The value of such close scrutiny of the subject's response to urea loading is obvious when one considers the stable relationship between parotid fluid and blood. There is, of course, no reason why other analyses cannot be performed likewise, since one can easily stimulate the parotid gland sufficiently to maintain a filled system.

Calcium and phosphorous determinations on parotid fluid have been made quite simple by the introduction of the AutoAnalyzer. The fundamental contribution of the machine to this determination has been in clarification of the dual nature of the precipitating substance, probably glycoprotein, that has heretofore prevented universally accurate inorganic phosphate determinations. Dialysis, coupled with trichloroacetic acid treatment, serves to produce consistently a crystal-clear filtrate-molybdate complex that contributes to highly accurate phosphorous determinations. It might be added here that in our laboratories we employ the AutoAnalyzer routinely in calcium and phosphorous determinations on ashed specimens of bones and teeth. This adaptation has been quite simple, the only requirements being that the dilutions be such that sample concentrations fall into ranges of linear response and that the standard solutions be modified to contain the same proportions of solvents as those employed in the final dilutions of the ashed specimen.

Whether a reducing substance being measured is "true glucose" has been the subject of such controversy with all so-called glucose methods that one hesitates to reintroduce the subject. Since one of our primary interests, however, is permeability characteristics of the acinar cells, the measurement of true glucose is of more than academic concern. For this reason, comparative analyses were undertaken in which the results of the AutoAnalyzer were compared to those of glucose oxidase determinations on the same samples. FIGURE 4 of our article shows that in blood serum the enzyme method gives lower glucose results and that the findings are essentially parallel. For parotid fluid we have found that by the enzyme method there is a measurable amount of "true

glucose" in all specimens and that by either method a response to glucose tolerance conditions is found. If one accepts the enzyme method as measuring only glucose, then one may conclude that slightly less than 50 per cent of the reducing substances measured by the AutoAnalyzer are glucose. From a clinical point of view, however, this is of no import. FIGURE 5 presents mean glucose values for 7 subjects who received 100 gm. of glucose orally; the parotid response is seen to mimic the serum results. As with the blood glucose tolerance procedures, we have found a much more predictable response in parotid fluid when the subjects are given intravenously 50 ml. of a 50 per cent glucose solution. The indications are clear that parotid fluid glucose tolerance tests can serve diagnostic functions.

LEONARD T. SKEGGS, JR. (*Western Reserve University, Cleveland, Ohio*): It is not difficult to understand why the first completely automatic system of colorimetric analysis was developed for use in clinical laboratories. The problems facing the clinical chemist are enormous. A moderate-sized laboratory routinely performs 12 to 18 different types of chemical analysis. Each of these procedures requires as many as 12 or more sequential operations. In addition, the number of samples involved is very large. Therefore, the task of the technician is great, for he may be required to perform 100 or more determinations per day, involving 1000 or more analytical steps without a mistake. Unfortunately this is not entirely possible, and many serious analytical errors are made. For economic reasons, duplicate determinations usually are not feasible, and mistakes sometimes can be very serious for the physician and the patient.

It therefore seems to me that the greatest contribution automation can make is to increase the degree of reliability of clinical laboratory methods simply by reducing to a minimum the opportunities for human error. The AutoAnalyzer has been very successful in this connection. Unfortunately there remain many procedures that are not yet possible with this equipment. Among them are some of greater difficulty, such as plasma protein, protein-bound iodine, cholesterol, and transaminase analysis. One of the remaining problems, therefore, is to provide automatic processes for these and other remaining materials and thus make a completely automatic clinical chemistry laboratory a reality.

EDWARD WAGMAN (*Chemistry Section, Research and Development Division, Bureau of Ships, United States Navy, Washington, D. C.*): The needs of the United States Navy for automatic chemical instrumentation are appreciable. The number of analyses that personnel on a ship must perform ranges through a fantastic array of quantitative procedures. For example, we analyze water for a variety of constituents such as chloride, phosphate, calcium and magnesium hardness, available chlorine, dissolved oxygen, pH, alkalinity, and chromate, or for such complex molecule impurities as lubricating or fuel oil. Sometimes we must analyze fuel oil for sea water contamination, lubricating oil for dilution, or a primary coolant water sample for the fission decomposition products strontium or iodine. On a submerged submarine, analysis of the atmosphere for oxygen, hydrogen, carbon monoxide, carbon dioxide, and fluoro-

carbon levels is performed routinely. Thus the procedural requirements for these analyses may vary from a simple color check to the complexities of an atmosphere analyzer embodying infrared, thermal conductivity, and paramagnetic principles.

Performance of these tests is done on shipboard by enlisted personnel who have had some preliminary training during a brief shore station school assignment or aboard ship. In either case, there is only time to teach the step-by-step procedure and give some assistance in such operational techniques as how to read a buret, fold a filter paper, or pipette out a sample. There is no professional chemist aboard who might provide the necessary background interpretation to resolve any shipboard test problem. The technician must depend on his own ingenuity to recognize and take corrective action when he encounters failure of an indicator to change color, deteriorated chemicals, interfering substances, or any other of the multitude of possibilities. Under such circumstances there should be obvious advantages in the adoption of simple straightforward automatic chemical instrumental methods.

Nevertheless, the decision to switch to an instrumental method must be weighed carefully. For example, in one of our chloride determinations the analytical procedure is simply the addition of silver nitrate and a check for the appearance of a silver chloride haze. This method is quick, easy to teach, fairly foolproof, and roughly accurate; however, a far more accurate automatic instrumental method may embody pumps, motors, timers, photocells, valves, recorders, and a variety of other equipment, all of it subject to some type of failure. I envy the utilization of automatic instrumentation ashore: if a plant instrument starts to misbehave, a telephone call to the plant laboratory provides immediate technical assistance. On a ship at sea no such procedure is possible. If the test procedure controls such a vital area as the quality of potable water or if it guarantees the absence of chloride in a stainless steel heat exchanger, one cannot wait until one reaches port to resolve the difficulty.

Obviously reliability is a major criterion of the acceptance of any instrumental method. However, we must emphasize also the ease of maintenance and minimal size and weight requirements. Instruments requiring a staff of trained mechanical, electronic, and electrical engineers to keep them in working order cannot and will not find general acceptance on our naval ships. Apparently what we need is some sort of realistic balance between the complexity of the perfect instrument and a greater simplicity in one that does an adequate job with minimal likelihood of trouble.

I have mentioned size and weight as additional criteria in our acceptance of any instrument. It is quite possible that here our needs may be different from those in industry, where these factors may not be so critical. Normally, when chemical test equipment goes aboard, it must be squeezed in somewhere, preferably in the immediate vicinity of the system being controlled. It is a rare ship, indeed, that offers available space. The situation cannot be improved by running sample lines to a centrally located instrument, since each sample line would have to penetrate numerous bulkheads. Each penetration would represent a possible weakening of the watertight integrity of the ship and therefore cannot be approved. In the end, the instrument must be fitted into

an existing, although not necessarily desirable, location. The problem may be complicated still further if at the same time place must be found for gallons of reagent and standard solutions. Obviously, the smaller the package, the more desirable it will be.

In conclusion, it is clear that there is ample need on our ships for continuous automatic instrumental methods, but that work should be directed toward modifying the equipment by reducing size and complexity, improving accuracy, simplifying means of calibration, minimizing maintenance, and increasing reliability. We feel that these goals are reasonable and are confident that progress will be made toward their achievement.

By technical manipulation of equipment the recorder response in the glucose procedure has been increased fourfold.

Further evidence that parotid fluid does contain glucose has been obtained by the administration of sodium tolbutamide and chlorpropamide to dogs. Parotid fluid glucose, measured enzymatically, invariably decreased markedly and, in some instances, completely disappeared.

WILLIAM NACOVSKY (*Consolidated Edison Company of New York, Inc., New York, N. Y.*): The ultimate objectives in instrument design for plant use should embrace the unit package that, once installed, is free from operating difficulties. Instruments should be designed with the best materials available for the job at hand and to operate under the most difficult situations anticipated with minimal service requirements. The vendor should leave the relatively clean, dry, comfortable ivory tower of his laboratory, where tests are usually made over a short period of time on synthetic samples, and base his designs upon results obtained when instruments are exposed to actual operating conditions.

To meet industry's requirements, the instrument should be (1) capable of reproducible accuracy and sensitivity over long periods of time; (2) easily maintained, preferably with interchangeable modules; (3) contained in an air-conditioned cabinet for control of adiabatic temperature and of dust conditions; (4) protected with devices to stop pumping of corrosive fluids in the event of instrument failure, preferably devices designed to activate an alarm; and (5) designed to permit automatic control of chemical feed requirements.

It is a well-established fact that instruments give satisfactory results in proportion to the degree of maintenance accorded them. To this end, the adequate implementing of instrumentation in a process industry requires the employment of qualified personnel with a comprehension of the work at hand and of the operating principles of the instruments to be used.

Technicians of this caliber are not generally available upon completion of their formal education. It behooves industry to fill the void between school and industry by indoctrinating these neophytes in the intricacies of the instruments to be serviced. Whenever possible, the assistance of instrument manufacturers should be made available to them for a better understanding of the operating features of specific instruments.

It should be noted, however, that the best qualified personnel for these functions frequently comes from the ranks of amateurs interested in specific phases of the instruments. When we observe the performance of these individuals,



the question naturally arises whether instrument wisdom is a function of environment at school or in the home.

From the point of view of utilization, automatic instrumentation has found substantial use in the chemical laboratory. When tests are made to differentiate between small changes in concentration of a material, it is mandatory that all components of the test be maintained at a constant level. Automation provides the answer by controlling quantity of reagents, time, temperature, and pressure for reaction with a specified amount of sample for test.

For the evaluation of water for use in high-pressure boilers, numerous procedures have been developed that are susceptible of being automated, including the very satisfactory silico-molybdate reaction for the determination of silica. With further refinements now in progress, it is believed that a suitable recording instrument for the monitoring of silica in water at such low concentrations as 10 parts per billion (ppb) will be made available.

Some forms of silica in water, however, do not respond to this procedure. Currently, this type of material is evaluated by solubilization of the nonreactive silica in spot samples under conditions of elevated temperatures and pressures. The need exists for the development of a procedure for the continuous measurement of *total* silica in boiler feed water and in the boiler water itself.

In the field of nuclear plant operations it is anticipated that many more questions pertaining to trace concentrations of elements will be raised. A direct procedure for the determination of chlorides in the range of 1 to 10 ppb would be most useful.

The incidence of good automation techniques in water testing already is apparent. It is confidently expected that continued improvements in automatic analytical equipment operated by competent personnel will provide the answers to today's unresolved problems.



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